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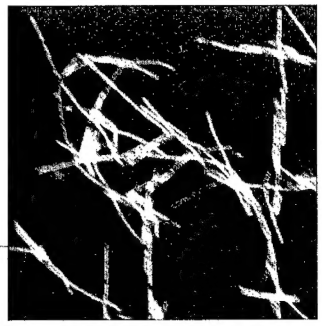


CONFERENCE ON

Anthrax

PROGRAM AND ABSTRACTS BOOK

June 10 - 13, 2001

St. John's College

Annapolis, Maryland, USA

4th International Conference on Anthrax

Program and Abstracts Book

June 10 – 13, 2001

St. John's College

Annapolis, Maryland, USA

Contents:

General Information

LOCATION

All scientific activities for the 4thInternational Conference on Anthrax will be held at St. John's College, Annapolis, Maryland, USA.

COMMITTEES

Organizing Committee
Arthur M. Friedlander, USAMRIID, Fort Detrick, Maryland
Les W.J. Baillie, DERA, Porton Down, United Kingdom
Bruce E. Ivins, USAMRIID, Fort Detrick, Maryland
Paul Keim, Northern Arizona University, Flagstaff, Arizona
Stephen H. Leppla, National Institute of Dental and Craniofacial Research, Bethesda, Maryland
Michèle Mock, Institut Pasteur, Paris, France
Susan L. Welkos, USAMRIID, Fort Detrick, Maryland

Local Program Committee – USAMRIID, Fort Detrick, Maryland
Marcia F. Baker
Patricia F. Fellows
Arthur M. Friedlander
Bruce E. Ivins
Stephen F. Little
M. Louise Pitt
Susan L. Welkos
Patricia L. Worsham

ACKNOWLEDGEMENT

The Organizing Committee would like to gratefully acknowledge the support of the U.S. Army Medical Research and Material Command.

REGISTRATION AND INFORMATION DESK

The registration desk will be open the following times for on-site registration, pick-up of preregistration materials, badge corrections and changes, and general information about the meeting and Annapolis.

Sunday, June 10	12:00 noon – 6:00 p.m.
Monday, June 11	7:00 a.m.– 6:00 p.m.
Tuesday, June 12	8:00 a.m.– 6:00 p.m.
Wednesday, June 13	8:00 a.m.–12:30 p.m.

SESSIONS

A badge is required for admission to all sessions.

Oral Sessions will be held in the Francis Scott Key (Key) Auditorium.

Posters will be held in the Great Hall located inside McDowell Hall. Posters are to be placed on boards by Sunday, June 10, 6:00 p.m., and will remain up throughout the meeting. Attendees are welcome to browse through the poster area during the day. During the official poster sessions, designated presenters will be available to discuss their posters. Formal Poster Sessions will be held Monday and Tuesday, 4:30–6:30 p.m.

NIGHT ON THE TOWN

Monday night, attendees will have the chance to enjoy Annapolis on their own. Organizers have arranged a number of dining reservations at local restaurants that are available for sign-up for those that would like to go out with other attendees. To participate, please sign-up at the registration desk. Group sizes are limited.

A candlelight walking tour of Annapolis will leave from Key Auditorium Lobby at 8:30 p.m. There is a $9 charge per person. If you would like to participate, please sign up at the registration desk by 5:00 p.m. Monday.

The Schooner *Woodwind* will have a tour available Monday from 6:30–8:30 p.m. See page 4 for details.

CONFERENCE PICNIC

Please join your colleagues and guests at the conference picnic on the Upper Playing Field the evening of Tuesday, June 12 at 7:00 p.m. There is no additional cost for this event.

ST. JOHN'S COLLEGE

St. John's College is a co-educational, four-year liberal arts college known for its distinctive "great books" curriculum. The college traces its origins to King William's School founded in 1696. The school was the first in the U.S. to prohibit religious discrimination when it was chartered in 1784. St. John's College is the third oldest institution of higher learning in the country – after Harvard and William and Mary.

ANNAPOLIS, SITES & TOURS

Annapolis, the capital of the state of Maryland, was first settled in 1649. From 1783 to 1784 it doubled as the capital of the United States. It is here that General George Washington resigned as Commander-in-Chief of the Continental Armies, and the Treaty of Paris was ratified, ending the war with Great Britain.

Annapolis today is often referred to as "sailing capital of the United States". A large number of sailing vessels call Annapolis homeport, and the United States Naval Academy resides two blocks from the town dock. It is home to the world's largest in-water sail and powerboat shows.

The downtown region offers an ample selection of waterfront dining and shopping in a historical setting. Local pubs offer music from modern to jazz. Walk south three blocks from St. John's campus to the waterfront.

Other activities you may arrange for on your own are:

Annapolis Tours
Three Centuries Tours offers walking tours conducted by guides in colonial attire. Discover the history of Annapolis and the tradition of the United States Naval Academy. Daily tours are at 10:30 a.m. (Visitor Center, 26 West Street) and 1:30 p.m. (Information Booth, City Dock). *Charge: $9*

Historic Annapolis Foundation Walking Tours
Offering two self-guided tours, "Historic Annapolis Walk with Walter Cronkite" and "African American Heritage Walking Tour," available at the Historic Annapolis Foundation Museum Store, 77 Main Street. Sunday–Thursday, 10:00 a.m.–7:00 p.m. and Friday–Saturday, 10:00 a.m.–10:00 p.m. *Charge: $5*

Naval Academy Guide Service
Guided walking tours of the U.S. Naval Academy are available Monday–Saturday, 9:30 a.m.–3:00 p.m., and Sunday, 12:30 p.m.–3 p.m. Tours meet at the Armel-Leftwich Visitor Center, 52 King George Street. *Charge: $6*

U.S. Naval Academy Chapel
"Cathedral of the Navy." Tiffany Studios designed many of the stained glass windows; a separate pew is dedicated to all POWs and MIAs.

Schooner Woodwind
74-foot classic wooden sailing yacht, *Woodwind* was designed for the Chesapeake Bay to provide exciting two-hour sails. Tours sets sail four times daily Tuesday–Sunday, 11:00 a.m.–1:00 p.m., 1:30 p.m.–3:30 p.m., 4:00 p.m.–6:00 p.m., and 6:30 p.m.–8:30 p.m. A Monday tour is available 6:30–8:30 p.m. All departures are from Pusser's Landing at the Annapolis Marriott Waterfront Hotel next to the City Dock. *Charge: $24 daytime weekday/$27 sunset and weekend*

Charles Carroll House
Home of Charles Carroll, the only Catholic to sign the Declaration of Independence and one of the wealthiest men in colonial America. Partially restored House, 18th century terraced gardens overlooking Spa Creek. Open 12:00 noon–4:00 p.m. *Charge: $5*

William Paca House & Garden
Georgian mansion built by William Paca, signer of the Declaration of Independence. Two acre Paca Garden features parterres, herb/vegetable area and fish-shaped pond. Open Monday–Saturday 10:00 a.m.–5:00 p.m., Sundays 12:00 noon–4:00 p.m. *Charge: $8*

City Dock
The colonial port once surrounded by warehouses, a ship carpenter's lot and taverns. It is presently the site of waterfront park, headquarters of the Harbor Master, and Visitors Information Booth.

Maryland State House Tours
Free walk-in tours of the first floor are given at 11:00 a.m. and 3:00 p.m. daily. The Welcome Center is open 9:00 a.m.–5:00 p.m. weekdays and 10:00 a.m.–4:00 p.m. weekends.

SCIENTIFIC PROGRAM & SCHEDULE OF EVENTS

SUNDAY, JUNE 10

REGISTRATION
Sunday, 12:00 noon – 6:00 p.m. | Key Auditorium Lobby

POSTER PLACEMENT
Sunday, 12:00 noon – 6:00 p.m. | McDowell Hall

OPENING RECEPTION
Sunday, 6:00 – 9:00 p.m. | Boat House
Complimentary drinks and light hors d'oervres

MONDAY, JUNE 11

CONFERENCE INTRODUCTION
Monday, 8:15 a.m. | Key Auditorium Lobby
ARTHUR FRIEDLANDER, USAMRIID, Ft. Detrick, MD

ORAL SESSION
Ecology and Epidemiology
Monday, 8:30 – 10:15 a.m. | Key Auditorium
Conveners: MARTIN HUGH-JONES, Louisiana State University, Baton Rouge, LA; PETER TURNBULL, Arjemptur Technologies, LTD., Porton Down, Salisbury, UK

Global Report, 2000
MARTIN HUGH-JONES, WHO Anthrax Research and Control Working Group and Louisiana State University, Baton Rouge, LA

The 2000 Livestock Epidemic in North-Central USA, Manitoba, & Ontario
HEIDI KASSENBORG, Minnesota Dept. Health, Minneapolis, MN

Anthrax in Wood Buffalo National Park, Alberta 2000
JONAH MITCHELL, Wood Buffalo National Park, Fort Smith, Alberta, CA; Brett Elkins, GNWT Department of Resources, Wildlife & Economic Development, Wildlife & Fisheries Division, Yellowknife, CA; and Pam Coker, Louisiana State University, Baton Rogue, LA

Epidemiological Applications of the *B. anthracis* Multi-locus VNTR Analysis in the Kruger National Park, South Africa
KIMOTHY SMITH, Northern Arizona University, Flagstaff, AZ

The Distribution of *B. anthracis* Strains in Two Countries, Italy and China
ANTONIO FASANELLA, Istituto Zooprophilico Sperimentale della Puglia e Basilicata, Foggia, Italy; and BINXIANG WANG, Lanzhou Institute of Biological Products, China

Summation
PETER TURNBULL, Arjemptur Technologies, Ltd., Porton Down, Salisbury, UK

COFFEE BREAK
Monday, 10:15 – 10:30 a.m. | Key Auditorium Lobby

ORAL SESSION
Classification, Identification, and Detection
Monday, 10:30 – 12:30 p.m. | Key Auditorium
Conveners: JOHN EZZELL, Diagnostics Systems Division, USAMRIID, Ft. Detrick, MD; PAUL KEIM, Northern Arizona University, Flagstaff, AZ

***B. anthracis* Identification and Taxonomy**
JOHN EZZELL, USAMRIID, Ft. Detrick, MD

Genetic Analysis of *B. cereus* and Relatives
ANNE-BRIT KOLSTØ, University of Oslo, Blindern, Oslo, Norway

AFLP Analysis of *B. anthracis* and other *Bacillus* sp.
PAUL JACKSON, Los Alamos National Laboratory, Los Alamos, NM

MIDI identification of *B. anthracis* and other *Bacillus* sp.
JEFF TESKA, USAMRIID, Ft. Detrick, MD

Dissection of *B. anthracis* into Distinct Genetic Subtypes
PAUL KEIM, Northern Arizona University, Flagstaff, AZ

LUNCH
Monday, 12:30 – 2:00 p.m. | Key Auditorium Lobby
Box lunches provided

ORAL SESSION
Cell Structure and Function
Monday, 2:00 – 4:00 p.m. | Key Auditorium
Conveners: MICHÈLE MOCK, Institut Pasteur, Paris, France; ANNE MOIR, University of Sheffield, Sheffield, UK

***B. anthracis* S-Layer: Anchoring and Dynamic**
AGNES FOUET, Institut Pasteur, Paris, France

The Exosporium
SARAH TODD, University of Sheffield, Sheffield, UK

Germination in *B. cereus*
ANNE MOIR, University of Sheffield, Sheffield, UK

Germination in *B. anthracis*
PHILIP HANNA, University of Michigan Medical School, Ann Arbor, MI

Germination of *B. anthracis* in Macrophages
CHANTAL GUIDI-RONTINI, Institut Pasteur, Paris, France

Discussion of presentations
ANNE MOIR AND MICHÈLE MOCK

POSTER SESSION I
Monday, 4:30 – 5:30 p.m. | McDowell Hall
Complimentary drinks

P1 – Ecology and Epidemiology (see pp. 18-20 for presentations)
P2 – Classification, Identification, and Detection (see pp. 20-23 for presentations)
P3 – Cell Structure and Function (see pp. 23-25 for presentations)

POSTER SESSION II
Monday, 5:30 – 6:30 p.m. | McDowell Hall
Complimentary drinks

P1 – Ecology and Epidemiology (see pp. 25-27 for presentations)
P2 – Classification, Identification, and Detection (see pp. 27-30 for presentations)
P3 – Cell Structure and Function (see pp. 30-32 for presentations)

NIGHT ON THE TOWN
Monday, 7:00 p.m.

TUESDAY, JUNE 12

ORAL SESSION
Genomics and Gene Regulation
Tuesday, 8:30 – 10:30 a.m. | Key Auditorium
Conveners: THERESA KOEHLER, University of Texas – Houston Medical School, Houston, TX; PAUL JACKSON, Biosciences Division, Los Alamos National Laboratory, Los Alamos, NM

The Expanding *B. anthracis* Toolbox
THERESA KOEHLER, University of Texas – Houston Medical School, Houston, TX

The Sequence of the *B. anthracis* Genome
TIMOTHY READ, The Institute for Genome Research, Rockville, MD

Identification and Expression of pX01 ORFs in other Species
JAMES PANNUCCI, Los Alamos National Laboratory, Los Alamos, NM

New Players in Anthrax Toxin Gene Regulation
ELKE SAILE, University of Texas – Houston Medical School, Houston, TX

The PlcR Regulon in Anthrax Pathogenesis
TÂM MIGNOT, Institut Pasteur, Paris, France

Silent ß-lactamase Genes of *B. anthracis*
YAHUA CHEN, University of Texas – Houston Medical School, Houston, TX

COFFEE BREAK
Tuesday, 10:30 – 11:00 a.m. | Key Auditorium Lobby

SPECIAL PRESENTATION
Tuesday, 11:00 a.m. – 12:00 noon | Key Auditorium

The Discovery of the Anthrax Toxin
HARRY SMITH, Medical School, University of Birmingham, Birmingham, UK

LUNCH
Tuesday, 12:00 noon – 2:00 p.m. | Key Auditorium Lobby
Box lunches provided

ORAL SESSION
Molecular Interactions of Anthrax Proteins
Tuesday, 2:00 – 4:00 p.m. | Key Auditorium

Conveners: R. JOHN COLLIER, Harvard Medical School, Boston, MA; STEPHEN LEPPLA, National Institute of Dental and Craniofacial Research, Bethesda, MD

Introductory Cartoon: Overview of Toxin Action
STEPHEN LEPPLA, National Institute of Dental and Craniofacial Research, Bethesda, Maryland

Dominant Negative Mutants of Protective Antigen: An Approach to Therapy of Anthrax
BRET SELLMAN, Harvard Medical School, Boston, MA

Crystal Structure of the Anthrax Toxin Lethal Factor
ROBERT LIDDINGTON, The Burnham Institute, La Jolla, CA

Edema Factor Structure and Function
CHESTER DRUM, Boston Biomedical Research Institute, Watertown, MA

Responses of the Macrophage to Lethal Toxin
MAHTAB MOAYERI, National Institute of Dental and Craniofacial Research, Bethesda, MD

POSTER SESSION III
Tuesday, 4:30 – 5:30 p.m. | McDowell Hall
Complimentary drinks

P4 – Genomics and Gene Regulation (see pp. 35-36 for presentations)
P5 – Molecular Interactions of Anthrax Proteins (see pp. 37-38 for presentations)
P6 – Immunoprophylaxis and Treatment (see pp. 38-42 for presentations)

POSTER SESSION IV
Tuesday, 5:30 – 6:30 p.m. | McDowell Hall
Complimentary drinks

P4 – Genomics and Gene Regulation (see pp. 42-43 for presentations)
P5 – Molecular Interactions of Anthrax Proteins (see pp. 43-45 for presentations)
P6 – Immunoprophylaxis and Treatment (see pp. 45-48 for presentations)

CONFERENCE PICNIC
Tuesday, 7:00 p.m. | Upper Field

WEDNESDAY, JUNE 13

ORAL SESSION

Immunoprophylaxis and Treatment I

Wednesday, 8:30 – 10:00 a.m. | Key Auditorium

Conveners: LES BAILLIE, DERA, Porton Down, Salisbury, Wiltshire, UK; BRUCE IVINS, USAMRIID, Ft. Detrick, MD

Vaccine Research at USAMRIID
ARTHUR FRIEDLANDER, USAMRIID, Ft. Detrick, MD

Protective Antigen Domains as Novel Anthrax Vaccine Candidates
HELEN FLICK-SMITH, DERA, Porton Down, Salisbury, Wiltshire, UK

Vaccine Research at DERA
LES BAILLIE, DERA, Porton Down, Salisbury, Wiltshire, UK

COFFEE BREAK

Wednesday, 10:00 – 10:15 a.m. | Key Auditorium Lobby

ORAL SESSION

Immunoprophylaxis and Treatment II

Wednesday, 10:15 – 11:30 a.m. | Key Auditorium

Conveners: LES BAILLIE, DERA, Porton Down, Salisbury, Wiltshire, UK; BRUCE IVINS, USAMRIID, Ft. Detrick, MD

Vaccine Research at Pasteur Institute
MICHÈLE MOCK, Institut Pasteur, Paris, France

Neutralizing Anti-PA Antibody Titer as a Correlate of Protective Immunity Conferred by Anthrax vaccine
SHAUL REUVENY, Israel Institute for Biological Research, Ness-Ziona, Israel

Engineering Antibody Therapeutics which Neutralize Anthrax Toxin
JENNIFER MAYNARD, University of Texas at Austin, Austin, TX

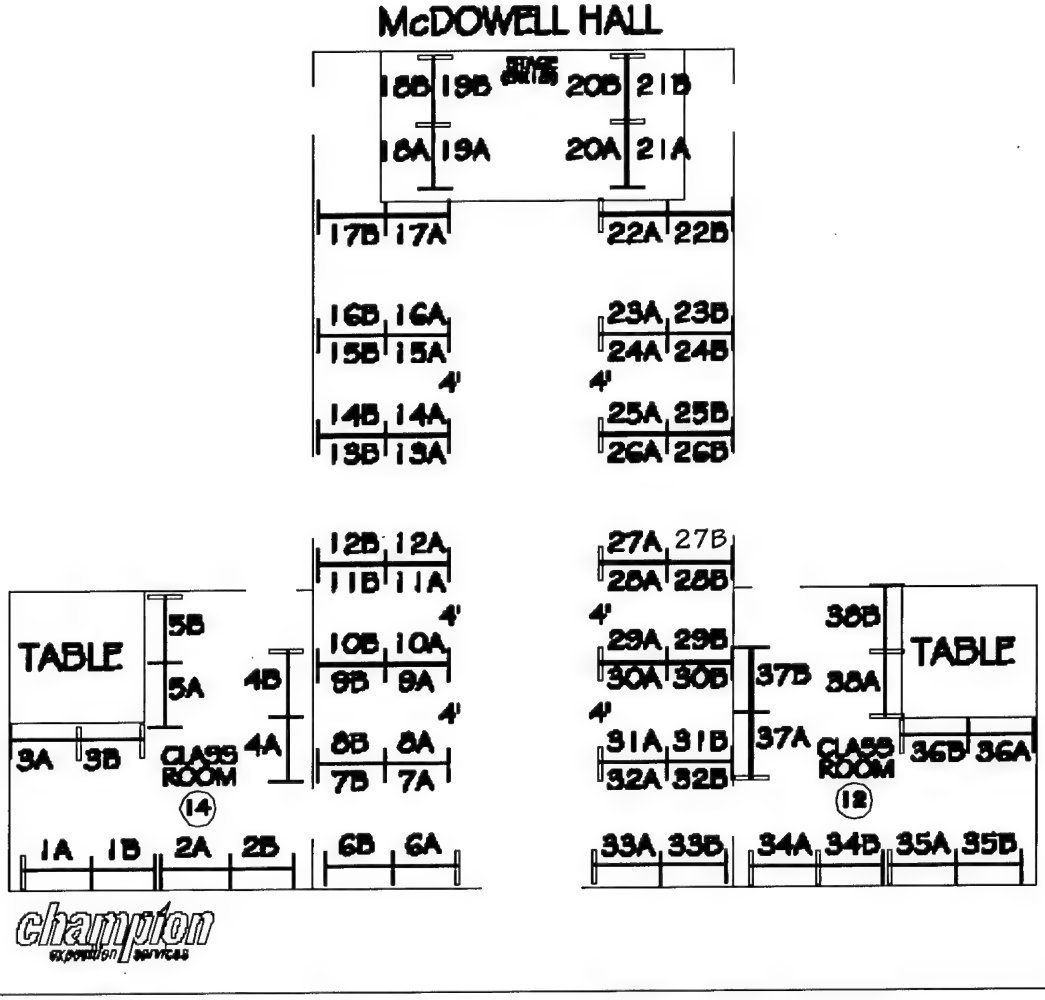
CLOSING REMARKS

Wednesday, 11:30 a.m. | Key Auditorium
ARTHUR FRIEDLANDER, USAMRIID, Ft. Detrick, MD

END OF CONFERENCE

THANK YOU FOR ATTENDING

ANTHRAX CONFERENCE POSTERS



POSTER BOARDS 39–43 ARE LOCATED
ON THE BALCONY

Abstracts

Ecology and Epidemiology

Monday, 8:30–10:15 a.m. | Key Auditorium

Global Report, 2000

M. E. HUGH-JONES

Louisiana State University, Baton Rouge, LA

Compared to previous years this seemed to be one of the more active while in reality the frequency of outbreaks may in fact be decreasing. What we are witnessing is an increase in awareness and thus more frequent reporting against a background of previous widespread under-reporting. This should be seen as a lesson to us all that full reporting is not as frightening as not knowing.

In North America an outbreak among wood bison was literally stumbled up upon by a survey team in the southern section of the Alberta Wood Bison National Park. It is accepted that the full scale of this outbreak was not found in spite of active searching with helicopters. In the midwest a series of outbreaks occurred from western Ontario across to southern South Dakota following extensive regional rains. And in Nevada three outbreaks occurred followed excavations of various kinds at three different sites and in retrospect disturbances of old cattle graves. This epidemiologic pattern of ground excavations, such as ditch clearing and bulldozing, being followed by livestock cases in areas where past cases were buried but have been free of disease for decades is common.

In Zimbabwe there were some 960 human cases (1.1% CFR) but only 188 reported livestock cases. The inversed human:livestock ratio has been ascribed to rural poverty and the selling of affected meat to others. In Norway there was the reportedly singular event of a addict dying from injecting himself with contaminated heroin. In Russia the decades of state-sponsored vaccination has resulted in many younger veterinarians never having seen a case and missing the occasional new case with subsequent severe public health repercussions. In Iran the disease is hypoendemic. They are perched on the cusp or attempting eradication in a situation mimicking Cyprus in 1957 where the disease was primarily of sheep & goats, not cattle. In Japan the disease reappeared having been absent for nine years. These and other events in the year 2000 will be presented and discussed.

Conclusion, we are still hobbled by the Pasteurean belief that anthrax is forever and only control is possible. No. If cases are found promptly, carcasses burnt, not buried, and the stock vaccinated for at least three years, the disease will not just be controlled but eradicated.

Anthrax in the North Central Plains-Summer, 2000: Implications for Animal and Human Health

H. KASSENBORG[1], T. BOLDINGH[2], L. SCHULER[3], P. SNIPPES[1], N. DYER[4], S. HOLLAND[5], G. LUTERBACH[6], G. SPEARMAN[7], R. DANILA[1]

[1]Minnesota Department of Health, Minneapolis, MN; [2]Minnesota Board of Animal Health, St. Paul, MN; [3]North Dakota Board of Animal Health, Bismarck, ND; [4]North Dakota State Veterinary Diagnostic Laboratory, Fargo, ND; [5]South Dakota Animal Industry Board, Pierre, SD; [6]Canadian Food Inspection Agency, Winnipeg, MB, CANADA; [7]Manitoba Department of Agriculture, Winnipeg, MB, CANADA

Livestock anthrax is endemic in the United States and Canada but is an infrequent diagnosis. From 7/17/00 to 9/25/00, livestock anthrax diagnoses were reported in North Dakota (ND), Minnesota (MN), Manitoba (MB), Ontario (ON), and South Dakota (SD). This report summarizes this outbreak which included diagnoses on new premises and human ingestion of *Bacillus anthracis*-contaminated meat. In ND, anthrax was diagnosed on 33 premises and in 180 animals; in MN, on 7 premises and in 31 animals; in MB, on 12 premises and in 44 animals; in ON, on 2 premises and in 14 animals; and in SD on 3 premises and in 10 animals. The majority of premises were in geographically adjacent regions of northwestern MN, northeastern ND and southern MB. In MN, anthrax had not been reported in the northern third of the state since recording began in 1909. Similarly, there were no records of previous anthrax diagnoses in the affected area in MB. Some areas received heavy June rainfall followed by a period of below normal precipitation and may have been a contributing factor in this outbreak. Lack of early veterinary recognition in MN contributed to the entry of a *B. anthracis*-infected animal into the human food chain. Control measures included livestock vaccination and the burning or burial of infected carcasses. This outbreak has significant human and animal health implications. First, this outbreak highlights the importance of considering the diagnosis of anthrax even on non-historical anthrax premises. Second, down animals should not enter the human food chain unless a specific non-human health hazard diagnosis can be made. Third, vaccination of livestock is recommended in all anthrax endemic areas.

Anthrax in Wood Buffalo National Park, Alberta, 2000

JONAH MITCHELL, BRETT ELKINS, PAMALA COKER

Wood Buffalo National Park, Box 750, Fort Smith, NWT X0E 0PO, Canada; GNWT Department of Resources, Wildlife & Economic Development, Wildlife & Fisheries Division, 600, 5102-5th Avene, Yellowknife, NWT X1A 3S8, Canada; Dept of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA

An outbreak of anthrax occurred in Wood Buffalo National Park during the months of July & August 2000, killing at least 103 bison, 2 black bears and a moose. Anthrax mortalities were found in three general areas of the Park. The index outbreak area occurred north of the Peace River with additional deaths clustered in an area south of the Peace River and in the Sweetgrass area. This was the 8th reported outbreak in the

WPNP since 1963 with the last being in 1992 when 32 bison died. The outbreak affected predominantly mature bulls, as on previous occasions. Surveillance was by helicopters and fixed wing aircraft. All carcasses found were marked and mapped using GPS but not treated or burned. In September, 19 diverse sites were revisited and soil sampled for spore contamination levels close to and distant from the now rotted carcasses. Surveillance of the Mackenzie and Hook Lake bison herds did not detect any evidence of anthrax. Approximately 3,000 sq. kms. of the park were closed to the public during the outbreak.

Epidemiological Applications of the *Bacillus anthracis* Multi-locus VNTR Analysis in the Kruger National Park, South Africa

K.L. SMITH[1], V. DE VOS[2], H. BRYDEN[2], M.E. HUGH-JONES[3], P. KEIM[1]

[1]Keim Genetics Lab, Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640 USA; [2]Scientific Services, Private Bag X402, Skukuza 1350, Kruger National Park, South Africa;[3]Special Pathogens Laboratory, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803 USA

The Kruger National Park, (KNP), in the Republic of South Africa, provides a unique opportunity to investigate interesting and unresolved aspects of the epidemiology of the disease anthrax. Anthrax is a disease of primarily mammals caused by the Gram-positive, rod-shaped, spore-forming bacteria *Bacillus anthracis*. Ninety-eight *B. anthracis* isolates from the KNP were analyzed using Multi-Locus VNTR Analysis. The environmental characteristics at the site of isolation were then compared for the genotype groups. We have shown that the two major anthrax genotypic groups in the KNP have unique environmental requirements. Additionally, the genotype groups were introduced as factors in an ecological study to determine the effect attributable to the anthrax genotype groups on anthrax mortalities. Results indicate that browsing species (such as kudu and nyala) have higher mortality rates than other species of ungulates in the KNP, as do animals near soil with high calcium and alkaline pH. A higher rate of host mortality and more stringent environmental requirements of one anthrax genotype group revealed two mechanisms which may have limited in this groups global distribution and genetic diversity.

Molecular Epidemiology of *Bacillus anthracis* in Italy

A. FASANELLA[1], K. L. SMITH[2], C. KEYS[3], P. COKER[4], P. KEIM[2], M. HUGH-JONES[4]

[1]Zooprofilattico Sperimentale della Puglia e della Basilicata, Foggia, ITALY; [2]Keim Genertics Laboratory, NAU, Flagstaff, AZ; [3]Keim Genetics Laboratory, NAU, Flagstaff, AZ; [4]Special Pathogens Laboratory, LSU, Baton Rouge, LA

The disease anthrax occurs sporadically in Italy, including Sicily and Sardinia. During the period 1988 to 1997, 41 human and 740 livestock cases were reported in 118 outbreaks. In a collaborative study, Multi-locus VNTR Analysis (MLVA) was used to genotype a collection of 42 Italian *Bacillus anthracis* cultures recovered from a variety of livestock species (cattle, donkey, goat, horse and sheep), the Italian equine and caprine vaccine, and a ca. 1940 vaccine from Eritrea (currently used to vaccinate cattle and sheep in Italy). These isolates were from the geographic range of anthrax occurrence in Italy including Abruzzo, Asmara, Basilicata, Campania, Puglia, Sardinia and Sicily. Results of the MLVA analysis revealed 6 new MLVA genotypes (allele combinations not seen previously in our worldwide collection). All isolates examined were clustered within the MLVA A1a branch, indicating that this group may be the "endemic strain" in Italy. Almost half of the isolates (20/42) matched the MLVA genotypic group 1. The Eritrea vaccíne strain, and the second largest group of isolates (9/42), most closely matched the MLVA genotypic group 3.

Molecular Epidemiology of *Bacillus anthracis* in China

B. WANG[1], K. L. SMITH[2], C. KEYS[2], P. COKER[3], P. KEIM[2], M. HUGH-JONES[3]

[1]Lanzhou Institute of Biological Products, Lanzhou, CHINA; [2]Keim Genetics Laboratory, NAU, Flagstaff, AZ; [3]Special Pathogens Laboratory, LSU, Baton Rouge, LA

Bacillus anthracis is distributed widely throughout China and continues to cause disease in humans and livestock in present day. During the period 1950 to 1970 human morbidity was 5 cases per million. In the last 20 years, China has reported between 1500 and 2000 human cases per year with approximately 4% mortality. Anthrax has been reported in 26 Chinese provinces, autonomous regions and municipalities, predominately in the western part of the country with Guizhou, Xinjiang, Guangxi, Yunnan, Sichuan, Tibet, Gansu, Inner Mongolia, Qinghai, and Hunan being the top 10. In a collaborative study, Multi-locus VNTR Analysis (MLVA) was used to genotype a collection of 193 Chinese *B. anthracis* cultures isolated in the period 1952 to1998 from a variety of sources (human patients, livestock, soil, etc.). These isolates were from the geographic range of anthrax occurrence in China. Results of the MLVA analysis revealed 21 new MLVA genotypes (allele combinations not seen previously in our worldwide collection). Additionally, isolates clustering within the MLVA A3b branch were found to be the most widely geographically distributed, indicating that this group may be the "endemic strain" in China. In contrast, a large number of especially diverse isolates were found in Xinjiang, the western-most province in China and historically, a major trade center on the Silk Route.

Classification, Identification and Detection

Monday, 10:30 a.m.–12:30 p.m. | Key Auditorium

Bacillus anthracis, *Bacillus thuringiensis* and *Bacillus cereus*: Janus-faces in Microbiology

ANNE-BRIT KOLSTØ, ERLENDUR HELGASON AND OLE ANDREAS ØKSTAD

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B. anthracis, B. thuringiensis and *B. cereus* are well established as closely related species with characteristic phenotypes for 2 of them. In most cases the distinction by classical criteria is therefore easy: *B. anthracis* is nonmotile, produces capsule, is usually non-hemolytic and does not produce phospholipase C. In addition it produces lethal and edema toxins. During sporulation *B. thuringiensis* produces intracellular crystals that are toxic to various insects. However, for the past few decades it has been known that genes encoding the typical phenotypic properties like the lethal toxins and capsular synthsis in *B. anthracis* and the crystal toxins of *B. thuringiensis* are located on plasmids – mobile genetic elements that may be lost or acquired under the right environmental conditions.

This has prompted more research on the genetic relationship between the three species.

Sequencing of 16S rRNA is the most widely used method for determination of the genetic relationship between bacterial species. By this criterion some *B. cereus* strains are very similar or identical to *B. anthracis* and some *B. thuringiensis* strains. Further analysis of the corresponding phylogenetic relationship by sequence comparisons of several other conserved genes and by multilocus enzyme electrophoresis confirms that *B. thuringiensis* and *B. cereus* should be regarded as one species, with *B. anthracis* as one lineage of this *B. cereus – B. thuringiensis* species.

One chromosomal difference between the *B. anthracis* lineage and the closely related *B. cereus – B. thuringiensis* strains is that the transcriptional regulator, PlcR, is mutated and nonfunctional in the *B. anthracis* strains, while about 50 genes appear to be regulated by PlcR in *B. cereus – B. thuringiensis* strains. Proteins like phospholipases and hemolysins are regulated by PlcR, and the inactive PlcR in *B. anthracis* may explain several of the phenotypic properties of this lineage.

The Relationship of *B. anthracis* to other Subgroup 1 *Bacillus* Species.

P. J. JACKSON, K. K. HILL, P. E. PARDINGTON, L. O. TICKNOR

Los Alamos National Laboratory, Los Alamos, NM

Based on classical methods and 16S rDNA analysis, the two closest *Bacillus anthracis* relatives are *B. cereus* and *B. thuringiensis*. It has been argued that, since there are no defining characteristics that fully distinguish between different *B. cereus* and *B. thuringiensis* isolates, these two species, and, by extension, *B. anthracis* should be considered a single species. Detailed phylogenetic studies based on Amplified Fragment Length Polymorphism (AFLP) analysis and supported by other molecular studies, also demonstrate that different *B. cereus* and *B. thuringiensis* isolates cannot be separated into two distinct groups. Such analyses demonstrate that some *B. cereus* isolates are more closely related to some *B. thuringiensis* isolates than they are to other *B. cereus* isolates. However, these analyses also reveal that all *B. cereus*, *B. anthracis* and *B. thuringiensis* isolates separate into at least five distinct phylogenetic groups. Phylogenetic differences among the different groups are greater than those normally seen among other closely related microbial species suggesting that each of these five groups could best be described as an independent species. All *B. anthracis* isolates so far analyzed map in a single cluster within a single branch of this phylogenetic tree suggesting that *B. anthracis* should be considered a unique species, separate from *B. cereus* and *B. thuringiensis*. Further analysis of the different phylogenetic branches reveals that isolates phylogenetically most similar to *B. anthracis* also share certain phenotypic traits with this pathogen. A high percentage of the *B. cereus* and *B. thuringiensis* isolates that map close to *B. anthracis* exhibit pathogenic traits. Many were isolated from contaminated food thought to cause "food poisoning" or were directly isolated from emetic or diarrheal samples. Others were cultured from infected tissue and were subsequently shown to be infectious in animal studies. Previously, laboratories dismissed *Bacillus* isolates cultured from such sources as environmental contaminants in the collected specimen. However, our results suggest that many such Bacilli may be infectious agents. Such pathogens must also be considered a possible source of genetic material that, when introduced into *B. anthracis*, may produce a pathogen that will be more virulent or difficult to eliminate from an infected host. A thorough study of these pathogens and comparisons of their expressed genes with those in *B. anthracis* may provide insights into the role of other virulence factors in *B. anthracis* pathogenicity.

Identification of *Bacillus anthracis* Using MIDI Whole Cell Fatty Acid Analysis.

J. D. TESKA[1], C. M. ALLAN[2], S. L. REDUS[2], S. R. COYNE[1], J. W. EZZELL[1]

[1]U.S. Army Med. Res. Inst. Infect. Dis., Fort Detrick, MD; [2]Sherikon, Inc., Fort Detrick, MD

The U.S. Army Medical Institute of Infectious Diseases, serving as a reference laboratory for the Department of Defense (DoD), has established an identification database for *Bacillus anthracis* using the automated bacterial identification system known as the MIDI (MIDI, Inc., Newark, DE). The MIDI system, based on the chromatographic analysis of methyl-ester fatty acids derived from bacterial membranes, was chosen for this evaluation based on its current application in regulated clinical laboratories. Strains of this gram-positive, aerobic, sporeforming bacterium, maintained within our frozen reference collection, were first screened by the gammaphage assay. Gammaphage sensitivity, indicating *B. anthracis*, was expressed by clear zones in a confluent lawn of growth caused by the lytic activity of the specific bacteriophage. Additional identity confirmation consisted of gene-target amplification with conventional PCR. Genomic DNA was extracted from the study strains with the QIAamp Tissue Kit (Qiagen, Inc., Valencia, CA). One nanogram of DNA from each sample was tested by conventional PCR using primers specific for the *B. cereus* group, protective antigen (PA) gene on plasmid pX01, and capsule (CAP) gene on plasmid pX02. After strain identities were independently confirmed by gammaphage and PCR, the project proceeded with the creation of the MIDI "anthrax

library". First, *Bacillus anthracis* strains were grown on 5% sheep blood agar plates at 35 ± 1 C for 24 ± 2 h and extracted according to the recommended five-step manual extraction procedure. Extracts were then run on a HP 68990 Series Gas Chromatograph and the resulting chromatographs analyzed with Sherlock software (MIDI, Inc., Newark, DE). The actual database library was generated from the same software. Once established, the "anthrax library" was further evaluated by re-running the isolates used to create the database, as well as assessing *Bacillus anthracis* isolates previously not seen by the instrument. The MIDI "anthrax library" demonstrated the ability to correctly identify *Bacillus anthracis*. Using this database, in conjunction with other libraries available from MIDI (i.e., CLIN4.0, TSBA4.0, and Bioterrorism Databases), *Bacillus anthracis* and closely related members of the Genus *Bacillus* can be distinguished.

Dissection of *Bacillus anthracis* into Distinct Types Using MLVA

P. KEIM[1], G. ZINSER[1], D. SOLOMON[1], C. KEYES[1], L. PRICE[1], A. KLEVYTSKA[1], K. SMITH[1], M HUGH-JONES[2].

[1]Northern Arizona University, Flagstaff, AZ USA, [2]Louisiana State University, Baton Rouge, LA USA

Our current understanding of *Bacillus anthracis* diversity is primarily due to polymorphisms at variable number tandem repeats (VNTRs) loci. The lack of other molecular variation suggests that *B. anthracis* changes very slowly in an evolutionary sense, or, that it is a recently emerged pathogen. Both are distinct possibilities. In order to better understand both global diversity and local epidemiology of anthrax, we have surveyed the *B. anthracis* genome and identified hundreds of potential VNTR loci. Nearly 40 VNTR loci have been converted into a multiple-locus VNTR analysis (MLVA) system and used to describe the phylogenetic structure from a representative set of diverse *B. anthracis* isolates. We find that the major clonal groups described previously are robust and well defined in this analysis. The three deepest branch points separate the 'A' group from the two distinct and deeply rooted B types (B1 and B2). The most numerous world-wide isolates are found in the 'A' group, but many representatives of the 'B1' and 'B2' are now known. Geographically, the 'A' types are found through out the world; the 'B1' isolates are primarily from southern Africa; the B2 types rare but found in Africa and in southern Europe including France. The use of VNTR loci for phylogenetic and epidemiological purposes presumes that they are rapidly evolving. In order to document this, we have performed a series of *in vitro* passage experiments. We established 423 separate lineages and transferred them 43 times to obtain nearly 500,000 generations. Five of the VNTR loci were observed to mutate. Mutational products included both insertion and deletions, though there were clear biases at particular loci. Highly diverse VNTR were most likely to mutate in this experiment, though at least one highly diverse locus did not. The fastest rate observed was ~$2x10^{-5}$ mutations per generation. We have used these mutational rate estimates to model epidemics and to estimate the number of generations involved in particular outbreaks. MLVA coupled with specific mutation rate estimates can be used to better understand *B. anthracis,* it world-wide distribution, as well as, its local transmission and ecology.

Cell Structure and Function

Monday, 2:00–4:00 p.m. | Key Auditorium

Bacillus anthracis S-layer: Anchoring and Dynamic

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The capsule is the outermost element of the cell wall. When *B. anthracis* does not produce its capsule, the S-layer, which overlays the petidoglycan is responsible for the highly patterned structure.

There are two abundant 94 kDa proteins, namely Sap (Surface array protein) and EA1 (Extractable Antigen 1), on the cell surface of *B. anthracis*. They are *B. anthracis* S-layer components. Both proteins harbor a standard Gram-positive signal peptide followed by two domains: the first, constituted of three SLH (S-Layer Homology) motifs, and the second, the protease resistant core, may be the crystallization domain.

There are at least 18 ORFs with SLH sequences in *B. anthracis*. Studies with *B. anthracis* S-layer proteins have shown that SLH motifs are responsible for cell wall binding. Polypeptides composed of the three SLH-motifs of EA1 or Sap are able to bind *B. anthracis* purified cell wall, in vitro, with similar K_D. Chimeric proteins combining the SLH domains of EA1 or Sap and the mature form of normally secreted proteins are anchored in their active form to the surface of the bacilli. The SLH domains do the same for the S-layer proteins.

This binding is to a secondary polymer covalently linked to the peptidoglycan. The analysis of the cell wall partner of the SLH domain led to the identification of a bicistronic operon (*csaAB*) involved in SLH anchoring. *csaB* mutant is unable to anchor S-layer proteins and also many other SLH-containing proteins. The only difference observed between the cell walls of the parental and the mutant strains is that the parental polysaccharide possesses pyruvate residues that are absent from the mutant. The *csaB* mutant bacilli have a twisted filamentous morphology due to a greatly reduced autolytic activity. The SLH-mediated cell wall anchoring mechanism is widespread among bacteria.

EA1 and Sap are major surface antigens, implying that they are both synthesized in vivo. In vitro, both proteins are also synthesized. Two lattices could co-exist or a co-crystallization could occur. The regulation of Sap and EA1 syntheses was studied using transcriptional fusions. *sap* expression precedes that of *eag*. Sap is a transcriptional repressor of *eag*. This differential expression leads to the replacement of Sap by EA1, the S-layers being sequentially present.

The *B. anthracis* S-layer has no influence on the LD_{50} in the animal models used. Nevertheless, capsule and the S-layer seem to have a cumulative effect increasing resistance to complement pathway-mediated defenses. Further analysis is required before the role of the S-layer becomes clear.

Proteins of the Exosporium of *Bacillus cereus*

S. J. TODD[1], S. CHARLTON[1], L. BAILLIE[2], A. MOIR[1]

[1]University of Sheffield, Sheffield, UNITED KINGDOM; [2]DERA, Porton Down, Salisbury, UNITED KINGDOM

The exosporium of *Bacillus cereus* UM20.1 has been isolated from mature spores. Proteins from the exosporium preparation were separated by SDS gel electrophoresis, and a number of associated proteins identified by N-terminal sequencing. Some proteins, including Immune Inhibitor A (InA) and GroEL, are looscly associated with the exosporium, as they can be removed by salt and detergent washes. Of the proteins remaining associated with the exosporium after such extensive stripping, many are likely to represent structural components. Nevertheless, two enzymes, alanine racemase and nucleoside hydrolase, were retained. This may explain the need for inclusion of alanine racemase inhibitors to achieve efficient alanine-stimulated germination in *B. cereus*. Attempts are being made to identify and inactivate the genes representing candidates for structural proteins. Several of these genes can be identified in the preliminary *B. anthracis* genome sequence (www.tigr.org).

Germination Genes of *Bacillus cereus*: A Background for Comparison

A. MOIR, P. J. BARLASS, J. BEHRAVAN, P. D. THACKRAY

University of Sheffield, Sheffield, UNITED KINGDOM

Bacillus cereus germinates rapidly in nucleosides, such as inosine or adenosine, or in L-alanine (the latter with an alanine racemase inhibitor). In addition, sub-germinal concentration of nucleosides stimulate alanine germination, and vice versa. The *gerA* family of operons in *B. subtilis* encode components of a germinant receptor, specific to particular germinants; the same is true in *B. cereus*. Transposon mutagenesis identified three loci, all *gerA* homologues, required for the response to specific germinants; the *gerI* and *gerQ* operons are both required for inosine germination, and the *gerL* operon for most of the L-alanine response. There are other homologues in the *B. cereus* genome, whose function has not been identified. Other germination associated genes include*gerN*, a single gene which encodes a likely sodium/proton antiporter important in inosine-stimulated germination, and the *gerP* operon, required for normal spore coat permeability to germinants. Close homologues of most, but not all, of the *B. cereus* germination genes are recognisable in the available, incomplete *B. anthracis* genome sequence data.

Establishment Stage of Anthrax Infections

P. C. HANNA

Univ Mich Med Sch, Ann Arbor, MI

Studies were performed investigating the establishment stages of anthrax infections: in vivo germination of the *Bacillus anthracis* endospore, macrophage-survival, growth and escape of the vegetative bacilli. Phagocytosis of all endospore types tested into RAW264.7 macrophage was both rapid and highly efficient. Phagocytosed *B. anthracis* endospores showed dramatic increases in germination, reaching nearly 100% in 30 minutes while endospores of non-pathogenic *Bacillus* spp. showed no increase in germination rates. Deconvolution of macrophage culture components indicated that a combination of amino acids and nucleic acids were required for full activation of anthrax endospores and none alone were sufficient. A mutant (MNR-1; macrophage non-responder-1) of *B. anthracis* was selected that was deficient in germination in macrophage cultures but fully capable of germination in bacterial media. Comparisons of *B. anthracis* Sterne, MNR-1, and related strains suggest a requirement for multiple germinant receptor genes, located on both the chromosome and on pXO1, are required for recognition of host-specific germinants and initiation of the infectious cycle. Individual assessment of theses receptors is ongoing. We found that newly vegetative bacilli escaped from the phagocytic vesicles of cultured Mf and replicated within the cytoplasm of these cells and were released from the Mf 4-6 hours after endospore phagocytosis. Genetic analysis indicated the toxin plasmid pXO1 is required for release from the Mf, while the capsule plasmid pXO2 is not. The transactivator atxA was required for Mf release suggests that Mf-release of anthrax bacilli, like other known virulence factors, may be atxA-regulated. The toxin complex genes were not required for release. Understanding switch from absolute metabolic dormancy of the endospore to growing virulent bacilli allows anthrax to be exploited as an effectual model for examining the earliest stages of bacterial infectious cycles.

Interaction of *Bacillus anthracis* Spores with Macrophages

C. GUIDI-RONTANI, M. LEVY, M. MOCK

Institut Pasteur, Paris, FRANCE

The fatal character of the infection caused by *Bacillus anthracis* spores results from a complex pathogenic cycle involving the transformation of a dormant spore into a vegetative cell. This step enables bacteria to actively proliferate and to synthesize their virulence factors such as toxins. Using immunofluorescent staining, confocal scanning laser microscopy and image cytometry analysis, we have investigated the fate of *B. anthracis* vegetative forms following germination within primary mouse macrophages. By using appropriate mutant strains we have shown that toxin activities are required in the survival of the germinated spores within primary macrophages. However, fluorescence imaging analysis strongly suggested that those germinated were unable to multiply within the macrophage. Neither capsule nor protein encoded by pXO2 contribute to this complex pathogenic process. These results contributed to the elucidation of strategies used by *B. anthracis* to survive within the host and to gain the external medium to proliferate.

Ecology and Epidemiology Poster Session I – P1

Monday, 4:30–5:30 p.m. | McDowell Hall

Board 1A. Studying the Effectiveness of Vaccination against Anthrax in Cheetah (*Acinonyx jubatus*) and Black Rhinoceros (*Diceros bicornis*), Two Endangered Species in the Etosha National Park, Namibia

P. TURNBULL[1], P. LINDEQUE[2], B. TINDALL[2], M. COETZEE[3], F. METTLER[3], N. DAVIS[4], R. BULL[4]

[1]Arjemptur Technology Ltd, Salisbury, UNITED KINGDOM; [2]Ministry of Environment and Tourism, Windhoek, NAMIBIA; [3]Central Veterinary Laboratory, Windhoek, NAMIBIA; [4]Naval Medical Research Center, Silver Spring, MD

It has recently been realised that cheetah are succumbing to anthrax in the Etosha National Park. As an endangered species, cheetah qualify for directed control measures, primarily vaccination. However, the cheetah has an unusual immune system associated with a remarkable lack of genetic diversity in the population. Therefore, the value of vaccination needs to be assessed scientifically. The additional danger anthrax poses to already endangered black rhino has long been recognized and vaccination campaigns have been carried out since the 1970s. However, the effectiveness of vaccination has never been monitored. In addition, vaccination is done by means of drop-out darts leaving it uncertain whether a dose, or complete dose, has been delivered. The work described was aimed at evaluating vaccination in cheetah and black rhino. Serum samples were drawn from 10 cheetah before vaccination with a single dose of the Sterne animal anthrax vaccine on 9.9.2000 and one and two months later. Serum samples were also taken from 5 vaccinated and one unimmunized rhino. The positive control for the cheetah was a lion bled on 22.11.2000 which had a naturally-acquired anti-PA titer of 1:12800. That for the rhinos was a horse vaccinated >20 times with the Sterne vaccine and which had an anti-PA titer of >1:25,600. Antibodies to cheetah and rhino immunoglobulins being unavailable, ELISAs were done using conjugated feline and equine antibodies respectively as the best relation alternatives. The sera were also used in passive protection studies. Half ml volumes of sera from the cheetah and rhinos were administered i.p. to 3 to 5 A/J mice (depending on serum availability). After 24 h, each mouse received 1.75 x 106 cfu of Sterne strain spores s.c. in 0.1 ml of PBS. Over a 2-week observation period, deaths were confirmed by culture as being due to the infecting *B. anthracis*. Anti-PA and anti-LF titers in the vaccinated cheetah and rhino never exceeded 1:800. However, in the passive protection tests, a level of protection, albeit variable in the different animals, was conferred by the sera of the vaccinated cheetah one month after the vaccination and this showed signs of decline at two months. Sera from four of the vaccinated rhinos conferred protection on 80-100 % of the mice. All the negative control mice receiving sera from unvaccinated animals died and all the mice receiving sera from the hyperimmunized horse lived. Only 3 of the 5 mice that had received serum from the lion with the high anti-PA titer survived, however. The protection results seemed to correlate poorly with the ELISA titers – a commonly reported finding in protection tests involving actively immunized animals.

Board 2A. Seroconversion in Bison(*Bison bison*)in Northwest Canada Experiencing Sporadic and Epizootic Anthrax

P. C. B. TURNBULL[1], J. RIJKS[2], I. THOMPSON[2], M. HUGH-JONES[3], B. ELKIN[4]

[1]Arjemptur Technology Ltd, Salisbury, UNITED KINGDOM; [2]Royal Veterinary College, London, UNITED KINGDOM; [3]Louisiana State University, Baton Rouge; [4]Department of Resources, Wildlife & Economic Development, Yellowknife, CANADA

Sera from bison (*Bison bison*) of two areas of northwest Canada which experience sporadic and epizootic anthrax were comprehensively examined with a view to gaining a better understanding of the epidemiology of an epizootic that occurred in wood bison in the Mackenzie Bison Sanctuary in 1993. The results indicate that sera from a substantial proportion of bison in the Sanctuary after the epizootic had substantial IgG and IgM antibody titres against the three anthrax toxin components, protective antigen (PA), lethal factor (LF) and oedema factor (EF) with significantly higher means than those of sera collected in the same region in the period 1986-1990. The implication is that a significant proportion of the bison survived *B. anthracis* infection during the epizootic. This contrasts with findings elsewhere in enzootically exposed wild herbivores, in which the observation of detectable antibodies in only a very low proportion of the population was interpreted as indicating that infection in herbivores usually leads to death. Although most of the sera collected before the 1993 outbreak had negative titres, or low titres regarded as possibly test anomalies, a few individuals had intermediate to high titres, particularly for anti-PA antibodies, indicating that bison in the Sanctuary were already being exposed to anthrax in the 1980s. High titres were found in sera from 4.5 to 6 month-old calves which had been removed when a few days old from wood bison dams in the Hook Lake area (which had experienced anthrax) to be raised in conditions under which they were presumed not to have been exposed to anthrax. In contrast, yearlings (1.5 years of age) caught and raised in the same conditions had significantly lower titres. It was thought that the explanation lay in maternal antibody still circulating in the calves. The results of serology support the indications from other studies that the epizootiology of anthrax in this particular ecosystem has interesting differences from what is generally encountered in other enzootic ecosystems.

Board 5B. Human Anthrax in the United States: A Descriptive Review of Case Reports, 1955-2000

D. A. ASHFORD[1], J. FLETCHER[2], P. BRACHMAN[3]

[1]CDC, Atlanta, GA; [2]Georgia Department of Health, Atlanta, GA; [3]Emory University, Atlanta, GA

Objectives. This study describes the epidemiology of human anthrax infections in the United States between 1955-2000. Methods. A retrospective case study of anthrax infections in humans occurring in the United States between January 1, 1955 and December 31, 2000 was conducted. The study population consisted of all cases of human anthrax reported to the Centers for Disease Control and Prevention during the specified period. Results. Between January 1, 1955 and December 31, 2000, two-hundred –thirty-seven (237) cases of anthrax infections in humans were reported. Confirmation of *B. anthracis* infection by a smear, culture, serology, or post-mortem exam was documented in 53% of the reported cases. Epidemiologic data and clinical findings were similar between

the confirmed and probable cases and were combined for the analyses. Infections occurred primarily in white males between 20-60 years of age and were most frequently associated with the goat-hair processing industry. Women, and especially children, were infected much less frequently. Most (72.4%) of the anthrax infections were associated with industry (industries involved in the processing of contaminated hair and hide products), 20.3% were agricultural (farmers and veterinarians following contact with infected animals or carcasses) and 2.1% were associated with contact with a contaminated commercial product. The cutaneous form of the disease occurred most frequently (95.3%), the inhalational form was rare (4.7%), and there were no cases of the gastrointestinal form reported. Clinical findings for cutaneous disease demonstrated a large number of cases with edema, erythema, pruritis and eschar formation at the site of infection. Most cutaneous cases were treated with oral and/or injectable penicillin. There was one fatality among the cutaneous cases. Eleven inhalational cases were reported over this period, of which nine were fatal. Individuals with inhalational infections reported a flu-like prodrome, followed by the acute onset of respiratory distress and diaphoresis. Often these patients were moribund on presentation to the health care facility. The fatality rate was high (81.8%) for these individuals despite treatment. There was a large decline in anthrax cases reported over the period of the study; most infections occurred in the 1950's and 1960's, then case reports dropped off dramatically. A case of human anthrax occurred in 2000 that was associated with a large epizootic in North Dakota. This is the first case since 1992.

Board 6B. An Outbreak of Cutaneous Anthrax in Changnyung, Korea, in 2000

H. B. OH[1], Y. M. PARK[1], J. K. PARK[1], J. S. HAN[1], W. K. SEONG[1], Y. H. SHIN[2]

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There was an outbreak of cutaneous anthrax on a small village located in Changnyung, southern Korea in July, 2000. Epidemiological study revealed 78 people had eaten the meat of cow died from anthrax in a farm. The cow was dead suddenly and slaughtered by residents. The meat was delivered among the residents and neighboring their relatives. As there was no one who had eaten raw meat, there was no sign of gastrointestinal anthrax. Several residents were involved in slaughtering the dead cow and contacted with the meat. Five cases of cutaneous anthrax were confirmed. All patients had experienced skin contact with meat during slaughtering and cooking. Among them, two people, a man of age 54 and an woman of age 72, died from meningitis 2-4 days after onset of skin symptoms. The other 3 persons showing only skin lesions on their hands and arms were recovered after hospitalization and treatment with antibiotics. The incubation period was 2 to 5 days and the case fatality of anthrax meningitis was 100%. The origin of the animal infection was not known. Twelve *Bacillus anthracis* were isolated from patient¡¯s blood, vesicular fluid, meat and temporary burial site. All isolates were analyzed by multiplex PCR for the identification of virulent *B. anthracis*. Serum samples were obtained from all the people who ate the meat. Tests for antibodies against protective antigen using EIA and western blot techniques were positive in patient subjects (in paired sera) with cutaneous anthrax. This is the first report, occurred in Korea, on fatal cutaneous anthrax followed by meningitis.

Board 8B. Human Ingestion of *Bacillus anthracis* - Contaminated Meat

H. KASSENBORG[1], R. DANILA[1], P. SNIPPES[1], M. SULLIVAN[1], R. LYNFIELD[1], M. WIISANEN[1], T. BOLDINGH[2], J. LINGAPPA[3], P. DULL[3], J. EZZELL[4], G. LUDWIG[4], T. LEWIS[4], T. ABSHIRE[4], R. CULPEPPER[4], A. FRIEDLANDER[4]

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Human gastrointestinal (GI) anthrax has not been previously reported in the U. S. On August 25, 2000, the MN Department of Health was notified that *Bacillus anthracis* was isolated from a steer in Roseau County. On July 24, the steer's owner had killed and gutted a cow that was unable to rise. It (carcass X) was sent to a custom processing-plant. Initial reports revealed human consumption of this meat; an investigation and public health response was initiated. Protocols and reagents developed by the Association of Public Health Laboratories (APHL) and CDC for bioterrorism preparedness were used to identify *B. anthracis* in carcass X samples, and to rule out contamination in carcasses processed subsequent to carcass X and from 24 processing-plant environmental samples. Meat consumption and illness histories were collected from persons who consumed or butchered carcass X or the first subsequently processed carcass (carcass A). Serum samples from 28 persons from these two groups were tested for antibody to the *B. anthracis* Protective Antigen toxin component at USAMRIID. Soil samples from the carcass X farm were obtained for testing. USAMRIID and CDC infectious disease specialists were consulted; draft ACIP guidelines were used for post-exposure prophylaxis recommendations. Two of six persons reported GI illness after consuming carcass X meat. One had a 3-day history of abdominal pain, diarrhea and fever of 102.3°; an acute serum sample only was collected. The second reported 1-day of diarrhea and had acute and convalescent serum samples drawn. Both recovered prior to antibiotic prophylaxis. *B. anthracis* was isolated from carcass X meat samples but not from any of the 7 subsequently processed carcasses nor from any environmental swabs. Of 28 serum samples collected, two sera had antibody of 1:100 and 1:400; with convalescent titers of negative and 1:100, respectively. Both symptomatic persons had negative titers. *B. anthracis* was isolated from 3/8 (38%) soil samples. Persons consuming carcass X or carcass A meat were recommended to receive six weeks of antibiotics; persons who consumed carcass X meat also received a 3-dose series of anthrax vaccine. This was the first use of APHL-CDC anthrax protocols and reagents and the ACIP anthrax post-exposure prophylaxis guidelines. Although two family members suffered GI illness after consuming carcass X meat, the diagnosis of GI anthrax was not confirmed; antibiotic use may have aborted antibody response. This report highlights several important lessons. A coordinated effort between local, state and federal agencies is critical for management of a public health emergency. Knowledge and experience gained can be used in the management and response

to a bioterrorism incident. Finally, a good working relationship between public health and the veterinary community is essential for surveillance of zoonotic diseases of public health importance.

Classification, Identification, and Detection
Poster Session I – P2

Monday, 4:30–5:30 p.m. | McDowell Hall

Board 10B. Study of Host Responses Induced by Anthrax Using Gene Array Technology

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Anthrax is a natural disease of herbivorous animals that can be transmitted to humans. A fatal form of human anthrax is caused by the inhalation of spores of *Bacillus anthracis*. Therapy for such exposures has been ineffectual probably because its detection/confirmation has been too late to administer antibiotics (high dose penicillin G) and perhaps vaccination to prevent relapse. Therefore early diagnosis is the most efficient way of preventing fatal Anthrax exposure. The molecular changes caused by this agent in the host have not been determined previously, and identifying genes altered by anthrax could be crucial for rapid and effective detection. Our current approach was to define the functional host response to anthrax exposure by studying gene expression changes in comparison to a library of known host responses to infectious and toxic agents. Using the gene array technology we have identified patterns of gene expression regulated by anthrax using human peripheral blood mononuclear cells (PBMC) in vitro and comparing them to fractionated buffy coat cells from monkey exposed to anthrax over various time periods. The in vitro exposures extended from 0-12 h while aerosol exposures to monkeys included 0, 24,48 and 72 h. Comparison of gene changes from in vitro and in vivo exposures revealed likely secondary effects of anthrax exposure. The kinetics of gene changes was quite dramatic. In the monkey samples, the changes in gene expression showed an interesting pattern with time of exposure to anthrax. Cytokine genes were induced by 24hrs of anthrax exposure but were at or below baseline levels at later time periods. Proteinases and apoptosis-inducing genes were upregulated in a time dependent manner. Many "symptom"-related genes were identified, especially at later time periods just prior to the onset of the shock-like signs. We are constructing a custom microarray containing the genes altered at the various time periods. This will provide an inexpensive means of mass evaluation of additional time periods of exposure as well as other factors so that we might better understand the course of this intractable illness, catalog gene patterns that could be used for early identification of anthrax exposure and identify new therapeutic strategies.

Board 12B. Nucleotide Sequence Analysis of *groEL* of *Bacillus anthracis*, *B. cereus*, *B. thuringiensis*, and *B. mycoides*

Y. H. SHANGKUAN, Y. H. CHANG

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Bacillus anthracis, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* are members of the *Bacillus cereus* group of bacteria, demonstrating widely different phenotypes and pathological effects. Previous studies have shown that *B. cereus* group strains demonstrate high levels (>99%) of 16S rRNA sequence similarity and that this method cannot differentiate between the four species. Data from recent studies suggest that an approximately 600-bp region of the chaperonin 60 gene, amplified by PCR with a single pair of degenerate primers, has utility as a potentially target for bacterial identification. 533-bp *groEL* nucleotide sequences were obtained for 53 reference strains representative of the diversity of the *B. cereus* group strains. A phylogenetic tree was constructed by the neighbor-joining method. Cluster analysis revealed three major groups. The intergroup sequence relatedness ranged from 94.8-98.2%. *Bacillus cereus* and *B. thuringiensis* strains were included in both Group I and II. There were two subgroups included in Group I. All but one *B. anthracis* belonged to Ia subgroup, and the 533 bases of *groEL* gene of these strains were 100% identical. The sequence relatedness between *B. anthracis* and *B. cereus* were between (99.3-99.6%) in this subgroup. There were three subgroups among Group II strains. Subgroup IIc included two *B. mycoides* and one *B. cereus* strain. Because of the rhizoidal colonial morphology the only *B. cereus* strain in subgroup IIc should be identified as B.mycoides. Group III included six *B. mycoides* strains. PCR primers were designed for the specific detection of *B. cereus* group bacteria. In addition, RFLP analysis of PCR-amplified *groEL* allowed separation of 53 *B. cereus* group bacteria into nine types. All 13 *B. anthracis* strains belonged to type A, which also included four *B. cereus* strains. Eight *B. mycoides* strains were separated into types H and I. In addition to rhizoidal colonial morphology and lack of motility, *B. mycoides* group could be distinguished from other *B. cereus* group bacteria by differences in groEL-RFLP profiles. Thirty-nine *B. cereus* strains isolated from soil in Taiwan revealed a high degree of heterogeneity in *vrrA*-RFLP test were assayed for groEL-RFLP. They were separated into five types identified by previous *groEL*-RFLP assay. In conclusion, analysis of the *groEL* gene of the *B. cereus* group bacteria provides a rapid and robust nucleotide sequence-based approach to identify and classify *B. cereus* group bacteria. The results indicate *B. anthracis* and some *B. cereus* strains belonged to the same lineage. Furthermore, *B. cereus* and *B. thuringiensis* strains were separated into two major groups and in the same group the strains of the two species were genetically highly related to each other.

Board 14B. A Specific, Sensitive and Rapid Assay For the Simultaneous Detection of Virulence and Genomic Targets of *Bacillus anthracis* Using the Smart Cycler®

D. E. BADER[1], M. M. VAILLANCOURT[2], M. BOISSINOT[2], F. J. PICARD[2], E. LEBLANC[2], M. G. BERGERON[2]

[1]Defence Research Establishment Suffield, Medicine Hat, AB, CANADA; [2]Infectious Diseases Research Center, Laval University, Quebec City, PQ, CANADA

Bacillus anthracis is a capsulating, aerobic, spore-forming, Gram-positive *Bacillus* that is pathogenic for humans and animals. Appropriate and timely management of the infection can be aided by molecular identification methods that are rapid, sensitive and specific. Taxonomically, *B. anthracis* belongs to the *Bacillus cereus* group, which includes *B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis* and *B. weihenstephanensis*. These species exhibit high levels of genomic DNA sequence similarity but *B. anthracis* can be distinguished within this group based on virulence genes located on two large plasmids, pXO1 and pXO2. Because these virulence genes can be potentially transferred to innocuous hosts by bioengineering, it would be desirable to develop a molecular identification method based on the simultaneous detection of plasmid and chromosomal targets. Following plasmid DNA sequence analysis of several *B. anthracis* strains, conserved sequence targets on both pXO1 and pXO2 were chosen. Sequence analysis of chromosomal DNA fragments from several relevant *Bacillus* strains, allowed the identification of a unique *B. anthracis* chromosomal polymorphism. A multiplex PCR assay based on these three different targets, was designed and optimized. The assay was performed using DNA purified from the *Bacillus cereus* group species and proved to be highly specific to all twelve *B. anthracis* strains tested. A sensitivity of approximately five genome copies was achieved after forty-five cycles of DNA amplification on the Smart Cycler® (Cepheid, Sunnyvale, CA) using SYBR Green I dye. Each of the three amplicons could be distinguished by analysis of melting curves generated by the instrument. This SYBR dye-based PCR assay is a specific, sensitive and rapid assay for the simultaneous detection of virulence and genomic targets of *B. anthracis*.

Board 16B. UK Anthrax Reference Service

A. ROBERTS, B. HALLIS, G. LLOYD

CAMR, Salisbury, UNITED KINGDOM

Anthrax is primarily a disease of herbivores. Humans acquire it because of contact with infected animals, animal products, or from materials infected with *Bacillus anthracis* spores. *Bacillus anthracis*, the causative agent of Anthrax, is a spore-forming, Gram positive, rod-shaped bacterium approximately 4? m by 1? m. It can survive for long periods, in its spore form, and is commonly referred to as an obligate pathogen. It is enzootic in many countries of Africa and Asia and it occurs sporadically in many other countries including USA, Canada and Australia. Anthrax infections are still considered important infections world-wide by both physicians and veterinarians. The pressure to develop "brown-field" sites has also raised the profile of environmental investigations required by current legislation. This presentation will cover the current CAMR Anthrax reference service activities and outline the developments made with biological and molecular detection strategies. Research activities have resulted in the development of new expression systems for the production of the individual anthrax toxin components (LF, EF and PA). These recombinant proteins have been used to develop rapid, sensitive and specific ELISAs for the detection of the individual anthrax toxin components. Assays have also been developed to monitor specific antibody production against the individual anthrax toxin components. Although Anthrax is rare in the UK, the risk of infection from a variety of sources must not be underestimated. The UK National Reference Service based at CAMR (Special Pathogens Reference Unit) undertakes clinical and environmental investigations. An overview of the reference services and the application of newly developed detection systems for use in monitoring clinical infections, vaccinees responses and environmental studies will be presented.

Board 18A. Training the Trainers: Building Bioterrorism Response Capacity to Rapidly Isolate and Identify *Bacillus anthracis*

C. MARSTON, B. DE, A. HOFFMASTER, L. LACLAIRE, S. BRAGG, R. WEYANT, L. BARDEN, S. PERRY, J. DELANY, T. POPOVIC

CDC, Atlanta, GA

As a result of renewed commitment and emergency funding for improving public health response to possible acts of bioterrorism, an admirable state of readiness for possible bioterrorism threats was achieved at the CDC in a very short period of time. Within the Meningitis and Special Pathogens Branch, a BSL-3 laboratory was developed and established with expertise for isolation, identification, rapid diagnosis, and molecular subtyping of *Bacillus anthracis*. CDC also established a nationwide public health laboratory response network, which will aid in the rapid detection of a bioterrorism event and identification of the agents used. This network consists of level A, B, C, and D laboratories with each level, beginning with A, having an increased capacity to handle and identify select agents. One of CDC's primary roles is to train level B laboratorians in the isolation and identification of threat agents. In the fall of 2000, the Bioterrorism Preparedness and Response Program sponsored four one-week long training sessions for public health laboratorians, focused on *B. anthracis*, *Brucella* spp, *Yersinia pestis* and *Francisella tularensis*. These sessions were conducted by staff from the Public Health Practice Program Office and National Center for Infectious Diseases, CDC and staff from the National Laboratory Training Network. The workshops were attended by 64 laboratorians representing all 50 states and the FBI. Using specific, recently developed criteria for the presumptive and confirmatory identification of *B. anthracis* by standardized and validated methods, Level B laboratorians were trained to rapidly confirm presence of *B. anthracis* in both clinical and environmental samples. In addition to training colleagues and level A laboratorians, workshop participants will be able to rapidly identify or rule out *B. anthracis* during a bioterrorism event or in a natural infection.

Board 19B. Use of Pulsed-Field Gel Electrophoresis to Investigate the Whole Genome Organization in the *Bacillus cereus*-Group of Bacteria

G. P. PATRA, M. NIEMCEWICZ, D. ZAKOWSKA, V. G. DELVECCHIO

University of Scranton, Scranton, PA

The genetic diversity of the *Bacillus cereus*-group of bacteria was studied using macrorestriction enzyme analysis followed by pulsed-field gel electrophoresis (PFGE). Several restriction endonucleases were tested, and the most discriminatory results were obtained with NotI, SmaI and SfiI. This technique confirms the extreme homogeneity of the *B. anthracis* genome organization. However, *B. anthracis* isolate were clearly differentiated from other members of the *B. cereus*-group. In addition macrorestriction/PFGE could differentiate isolate of *B. cereus*, *B. thuringiensis* and *B. mycoides* and thus can be useful for epidemiological and genome plasticity investigation of these bacteria.

Board 21B. Microarray for Genome-Wide Analysis of *Bacillus anthracis*

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[1]DSO National Laboratories, Singapore, SINGAPORE; [2]National Defense Research Establishment,, Umea, SWEDEN

The current method for detection and identification of *B. anthracis* uses PCR methods that target a chromosomal fragment, Ba813, and virulent-determinant genes carried by the two plasmids (pX01 and pX02). It has been postulated that virulent strains could spontaneously cure one or both of its plasmids, rendering it avirulent. The chromosomal marker has recently been found in other environmental *Bacillus* strain, thus reducing the confidence in its usage for identification. The identification of individual strains within the species is further hindered by the lack of polymorphism within the species. Identification of individual strains or typing of *B. anthracis* strains is important for epidemiological study and for following the evolution of the pathogen. The aim of our study is to explore the use of DNA microarray to perform a genome-wide screening for novel biomarkers for identification of *B. anthracis* and for strain typing within the species. An anthrax genome chip was fabricated with a shot-gun library of *Bacillus anthracis* Ames. Hybridisations of the chip with the genome of various strains reveal gene fragments that could potentially be used for identification and typing. From this chip, 536 potential biomarkers that show differences in distribution among *Bacillus* species or among the *B. anthracis* strains studied were identified. The clones of interest are in the process of sequence determination and the presence or absence of some biomarkers in each strain was confirmed by Southern Blot. Our poster will discuss the approach employed and the preliminary results obtained.

Board 22A. Use Of Immune Magnetic Separation And Pcr For *Bacillus anthracis* Detection In Soil Samples

E. EREMENKO[1], E. AFANASYEV[2], V. EFREMENKO[2], A. ABGARYAN[2], O. TSYGANKOVA[3], E. ZHDANOVA[2], N. SARKISOVA[2], I. ZHARNIKOVA[2]

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Express analysis of soil samples for presence of *B.anthracis* spores may be a problem because of huge contamination of such material by related saprophytic bacilli and minimal concentration of pathogen. Direct use of immunofluorescent assay, ELISA or even PCR often is not sufficient sensitive and specific for *B.anthracis* detection in this case. Immune magnetic separation is powerful technique for bacteria concentration and in combination with PCR dramatically increases sensitivity of assay. We had designed composite magnetic beads based on alum-silica loaded with adsorbed antibody to spore antigens of *B.anthracis*. One more technical approach, which increased specificity and sensitivity, was using of selective medium for spore outgrowth. The medium contained nutritional broth, polymyxin M sulfate and trimethoprim. Analysis composed of 3 consecutive steps: i - exposure of immune magnetic beads with samples; ii -washing and transfer of beads using magnetic rod into liquid selective medium for *B.anthracis* followed by incubation during 1,5 h at 37 oC; iii - boiling during 1 h, centrifugation at 10000 g - 15 min and using of supernatant for PCR. Amplification was performed with primers for cap B gene sequence (pXO2), pag (pXO1) [Tuchkov et al., 1994] and chromosomal sequence Ba 813 [Patra et al., 1996]. In model experiments with water extracts from soil samples artificially contaminated with *B.anthracis* spores this assay had sensitivity about 50 spores per ml. All analysis took not more then 6 h. In some *B.anthracis*-free soil samples PCR with primers to pag and Ba 813 gave false-positive results. These data ties up with reported by Bohm et al. (1996) and Ramisse et al. (1998) about presence of some *Bacillus* sp. in soil, which produced amplicons of same size as *B.anthracis*.

Board 24A. Elimination of *Bacillius anthracis* from Milk During Pasteurisation Followed by Fermentation with Lactobacilli

N. BURAVTSEVA, V. PROSKURINA, E. EREMENKO, V. YAROSCHUK, L. LISOGORA

Stavropol Research Antiplague Institution, Stavropol, RUSSIAN FEDERATION

B.anthracis can excrete from the organisms of animals with the aid of milk, saliva, urine and excrements in different periods starting with the moment of infection and during the whole period of illness. Milk from the farm with its further contamination by means of pathogenic organisms in spore and vegetative forms in doses 10 4– 10 6 cells in 1ml of milk, was used in experiments. Milk was spread in thermostable flasks – 100ml in each. Then the milk samples were being pasteurized for 30 min at the temperature of 65°C and underwent thermal treatment duration of 30 min – 2 hours at the temperature of 95°C. Then 3-5 % of milk ferments made of Lacto*Bacillus* acidophilus and L. bulgaricus were added to the cooled to the

acidophilus and L. bulgaricus were added to the cooled to the temperature of 35-37°C milk. After that the samples were situated into thermostat for incubation at the temperature of 37°C for the period of 18-20 hours. The viability of *B.anthracis* was checked with the help of method, when they were sowed on agar plates with differential-diagnosing medium. The experiments showed that thermal treatment or fermentation apart were not enough for complete extermination of microbe (the death of spores 21-62 %), when the spores *B. anthracis* fell into milk. At the same time thermal treatment (95°C) during 1 hour followed by fermentation was effective. After contamination of milk by vegetative form of microbe, as it is in natural conditions, even signal ferment is enough for milk disinfecting. Thermal treatment of milk at the temperature of 95°C during 30 min was not enough for complete extermination of vegetative forms of pathogenic organisms in milk. Pasteurization of milk followed by fermentation with the aid of milk ferments leads to complete elimination of *B.anthracis* from milk.

Board 26A. Purification of DNA for PCR from *Bacillus anthracis* Spores in Soil Samples

S. R. COYNE, P. D. CRAW, J. D. TESKA, J. W. EZZELL, D. R. SHOEMAKER, E. A. HENCHAL, F. K. KNAUERT

USAMRIID, Fort Detrick, MD

Preparing DNA for PCR from *Bacillus anthracis* spores in soil samples is particularly challenging. We previously determined that digestion with Proteinase K and mechanical agitation in the presence of glass beads were critical to disrupt spores and release DNA. In addition, we found that it was necessary to treat the soil sample with substances known to inactivate or remove inhibitory and degradative contaminants in order to produce template of sufficient quality to support PCR. Depending on the soil type, this decontamination process was sometimes insufficient and we found it necessary to further process the sample using the Life Technologies, Inc., GlassMAX or the Qiagen QIAamp Tissue kits. We tried to simplify the procedure by replacing the contaminant removal process with the Schleicher and Schuell IsoCode paper procedure but found that it was insufficient by itself to remove all PCR inhibitory and degradative components. However, we did find that if we used the Schleicher and Schuell procedure in combination with incubation of the sample in polyvinylpolypyrrolidone (PVPP) and Chelex 100, we were able to increase the amount of soil we could prepare per sample from 100 mg to 250 mg. Thus, we were able to simplify and increase the overall sensitivity of the procedure.

Cell Structure and Function Poster Session I – P3

Monday, 4:30–5:30 p.m. | McDowell Hall

Board 28A. Spore Coat and Germination Properties of *Bacillus anthracis* Spores

A. I. ARONSON

Purdue University, West Lafayette, IN

We have initiated a study of the structural and germination properties of *B. anthracis* spores with the intent of finding targets for spore inactivation. While the overall sporulation process of *B. anthracis* is probably identical to that of *Bacillus subtilis*, there are differences in the kinases involved in initiation and in the spore coat protein profile. The genome of *B. anthracis* appears to lack several *B. subtilis* spore coat proteins genes which encode polypeptides found primarily cross-linked in the outer coat. *B. anthracis* (and *Bacillus cereus*) spore coat extracts, however, contained atleast two major coat proteins not found in *B. subtilis*. One of these designated CotBc (ca 13kDa) is invariant in relative amount with the strain and medium. It appears to be at or near the spore surface since it was digested by agarose-linked trypsin treatment of intact spores. There is atleast one other novel coat protein which varies in amount with the medium and conditions of sporulation. Its relative content changed 2-3 fold when grown and sporulated in a yeast extract-glucose medium at 37*C in the presence of 5% CO2 as compared to spores formed at this temperature in the absence of CO2. No such variation was found for spores produced in brain heart infusion (BHI) medium. In addition, an S-layer-like protein was present in extracts of well washed spores but was absent when the spores were purified through a density gradient. Germination rates in L-alanine plus inosine at 25*C for all *B. anthracis* strains tested were about 15% of the rate for *B. cereus* 569 or T. The rates increased about 5 fold at 37*C for *B. anthracis* in contrast to only a 30% increase for *B. cereus* spores. *B. anthracis* spores responded hardly at all to only inosine or L-alanine, again in contrast to *B. cereus* spores. There was always a larger fraction of superdormant spores in the *B. anthracis* germinated population (5-10% versus

Board 30A. Studies of Conditions for Spore Germination and Vegetation In vitro and In vivo of some Subcultures of *Bacillus anthracis* 1(so)

O. TSYGANKOVA[1], E. EREMENKO[2], N. SARKISOVA[2]

[1]Stavropol Research Antiplague Institution, Stavropol, RUSSIAN FEDERATION; [2]Stavropol Research Antiplague Institute, Stavropol, RUSSIAN FEDERATION

The *Bacillus anthracis* strain 1(SO) and its subcultures previously selected as being phage-resistant and showing variability in a wide spectrum of properties [O.I.Tsygankova, E.I.Eremenko, 1999]. We studied the capability of the subcultures for spore germination and vegetation on the standard 9AT minimal medium [E.I.Eremenko, 1986], on Hottinger agar and its modifications with 1% of sodium bicarbonate, or with horse inactivated serum, or with both components taken together. The subcultures were incubated at 37 0C in an air atmosphere or in the anaerostat in an atmosphere with 20 or 50% of are substituted for CO2. Characteristics of some of the subcultures exhibiting varied capability for spore germination and vegetation under different conditions are presented in Table 1. The peculiarities of spore germination of subcultures 5 and 16 allow us predict isolation of such subcultures from populations of various strains by selection of newly formed colonies on bicarbonate-free media after additional incubation in an air atmosphere preceded by cultivation for 24-48 h in a 25-50% CO2 atmosphere and marking colonies formed at the first stage. Subcutaneous inoculations of white mice with 103 spores of subcultures 5 and 16 did not cause the death of animals. A culture isolated from a mouse inoculated with subculture 16 and killed after 10 days from the site of inoculation on the third passage produced variant 16 (white mouse 258). Unlike subculture 16, variant 16 (white mouse 258) inoculated on serum-bicarbonate agar in the form of a vegetative culture

showed growth in a 25% CO2 atmosphere and inoculated intraperitoneally in the form of spores or vegetative cultures formed long chains of bacilli with very pronounced capsules which were not found in blood and parenchymal organs for 24 h (period of observation). Studies of the cultures by PCR using primers pag 67-68, cya 25-26, lef 3-4 and cap C 57-58 [Ramisse et al., 1996] revealed the presence of all appropriate genes in variant 16 (white mouse 258), whereas subculture 16 showed the presence of gene cap C only. The probable absence of plasmid pXO1 with gene ger X located in it may be treated as the cause of disturbed spore germination in vivo and on media simulating to some extent these conditions. On the other hand, subcultures 15, 23 and 24 showing the absence of genes responsible for toxin-production grew well in similar conditions.

capability of subcultures of strain 1(SO) for vegetation under different conditions

Subcultures	Growth on media					Vegetation in vivo (intraperitoneal inoculation with spores)	Vegetation in vivo (intraperitoneal inoculation with vegetative cells)
	9AT	Bicarbonate-free		With bicarbonate			
		In an air atm.	In a 25%-50% CO2 atm.	In an air atm.	In a 25%-50% CO2 atm.		
5	+/-	+	-	-	-	no inoculation	no inoculation
11	-	+	-	-	-	no inoculation	no inoculation
15		+	+	-	+	no inoculation	no inoculation
16	+/-	+	-	-	-	-	-
16 w/m/258	not tested	+	+/-	-	-	+/-	+
23	+	+	+	-	+	no inoculation	no inoculation
24	+/-	+	+	-	+	no inoculation	no inoculation

Board 32A. Characterisation of the Exosporium of *Bacillus anthracis*

C. REDMOND[1], L. BAILLIE[1], S. HIBBS[1], A. MOIR[2], S. CHARLTON[3]

[1]DERA, Salisbury, UNITED KINGDOM; [2]University of Sheffield, Sheffield, UNITED KINGDOM; [3]CAMR, Salisbury, UNITED KINGDOM

The exosporium represents the primary physiological barrier between the spore and the environment. It is the first surface encountered by the host during the infective process and may play a role in virulence. To better understand the biology of this structure we have characterised by SDS PAGE the chemical nature of the exosporium of *Bacillus anthracis* and compared it to that of the closely related *Bacillus cereus*. Early electron microscopy studies showed the exosporium to have a hexagonal close packed crystal lattice structure (Gerhardt and Ribi, 1964, Beaman et al., 1971), consisting of a paracrystalline basal layer and a hair-like outer region (Gerhardt and Ribi, 1964; Hachisuka et al., 1966; Kramer and Roth, 1968; Moberly et al., 1966). Studies of the exosporium of *B. cereus* have shown it to be biochemically complex (Matz et al., 1970; Charlton et al., 1999). Using techniques developed in house we have been able to extract and characterise the protein profile of the exosporium of the highly virulent Ames strain of *B. anthracis*. Individual proteins are being identified by combination of N-terminal sequencing and access to the preliminary *B. anthracis* genome sequence (www. tigr.org). The role of these proteins, if any, in the infective process will be discussed. References Beaman, T.C., Pankratz, H.S., and Gerhardt, P. (1971) Paracrystalline sheets reaggregated from solubilized exosporium of *Bacillus cereus*. Journal of Bacteriology, 107 (1): 320-324. Charlton, S., Moir, A.J.G., Baillie, L., and Moir, A. (1999) Characterisation of the exosporium of *Bacillus cereus*. Journal of Applied Microbiology, 87: 241-245. Gerhardt, P. (1967) Cytology of *Bacillus anthracis*. Conference on Anthrax. In Federation Proceedings, 26 (5): 1504 – 1517. Gerhardt, P., and Ribi, E. (1964) Ultrastructure of the exosporium enveloping spores of *Bacillus cereus*. Journal of Bacteriology, 88 (6): 1774-1789. Hachisuka, Y., Kojima, K., and Sato, T. (1966) Fine filaments on the outside of spores of the exosporium of *Bacillus anthracis* spores. Journal of Bacteriology, 91 (6): 2382-2384. Kramer, M.J. and Roth, I.L. (1968) Ultrastructural differences in the exosporium of the Sterne and Vollum strains of *Bacillus anthracis*. Canadian Journal of Microbiology, 14: 1297-1299. Matz, L.L, Beaman, T.C., and Gerhardt, P. (1970) Chemical composition of exosporium from spores of *Bacillus cereus*. Journal of Bacteriology, 101: 196-201. Moberly, B.J., Shafa, F., and Gerhardt, P. (1966) Structural details of anthrax spores during stages of transformation to vegetative cells. Journal of Bacteriology, 92: 220-228.

Board 34B. Phagocytosis of Anthrax Spores by Macrophage-like Cells

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B.anthracis is known to produce a three-component toxin, the action of which determines all the symptoms of the infectious disease caused by this microorganism [Stanley, J.L., Smith, H.,1961; Leppla, S.H. 1988, 1995; Hanna, *et al.*, 1993; Hanna *et al.*, 1999; Sirard, G-C., *et al.*, 1996, Guidi-Rontani, C, *et al.*, 1999]. Recently, researchers have given special consideration to the early stages of interaction between the pathogen and macrophages [Guidi-Rontani, C, *et al.*, 1999; Hanna, *et al.*, 1999]. It has been established that spores are captured by macrophages and germinate inside the phagosome. The spores begin to synthesize the components of the anthrax toxins at the early stage of germination.

We have studied the process of phagocytosis of *B.anthracis* spores and factors that affect this process. Studies were performed by using the macrophage-like J774A.1 as a model; *B.anthracis* strains STI-1, Cinkovsky 1 and 81/1, with different degrees of virulence (LD_{50} = 10^5, 10^2 and 1-3 spores, respectively), were used as infecting agents. Survival of bacterial cells was estimated by CFU.

Our studies demonstrated that after phagocytosis of the spores by the macrophages, the spores germinate inside the macrophages and become vegetative cells. In preliminary experiments, the infecting dose was selected. Both the lysis of bacterial cells and their multiplication inside macrophages were observed at a spore/macrophage ratio equal to 10:1. At a higher ratio of spore/macrophage (100:1), effective multiplication only of the pathogen in the macrophage-like cells was observed.

The time course of survival of various strains in the macrophages was investigated during the 24 hour period. The numbers of viable organisms slowly diminished during the 4-6 hour period after phagocytosis and were drastically reduced by 8

hours after phagoytosis; it remained constant at this level over the remaining period of observation (24 hours). Moreover, we demonstrated a significantly higher efficacy of the macrophage infection by spores of the vaccine strains as compared with the spores of virulent strains. The reverse dependence was clearly distinct: the higher was the virulence of the strain, the lower was the efficacy of macrophage infection.

The effects of anti-PA and anti-LF antibodies on the phagocytosis of the virulent strains were also investigated. The results demonstrated that antibodies drastically reduce the survival of bacterial cells inside macrophages (phagocytosis). It is suggested that anti-PA and anti-LF antibodies contribute to the completion of phagocytosis at early stages of their germination in the macrophage phagosome.

Board 35B. Anthrax Nutrient Triggered Germination

M. WEINER, P. HANNA

University of Michigan Medical School, Ann Arbor, MI

Anthrax manifests itself quickly in infected mammals and results often in lethal infections that are difficult to treat because of the sudden onset of disease, the first symptom of which is often death. *Bacillus anthracis* endospores are the infectious particles that, once inside of a host, germinate rapidly into vegetative bacilli that are then capable of producing anthrax in an infected individual. A variety of germinant molecules trigger receptor mediated germination of *B. anthracis* spores by binding to specific receptors. *B. anthracis* has homologs of members of the *ger* family of germination operons that are necessary for nutrient triggered germination in other *Bacillus* species. Putative germination receptors encoded within the *ger* operons are packaged in the spore, and initiate germination within the first few minutes of infection in the presence of an appropriate germinant. Unlike several non-pathogenic *Bacillus* species, *B. anthracis* endospores show enhanced germination within macrophages. Identifying the germination receptors of *B. anthracis* and their corresponding germinant molecules may provide effective targets for preventing newly acquired infections.

Board 37B. *B. anthracis* Intracellular Survival and Escape: New Virulence Factors

T. C. DIXON, P. C. HANNA

University of Michigan Medical School, Ann Arbor, MI

This study describes early, intracellular events occurring during the establishment phase of *Bacillus anthracis* infections. Anthrax infections are initiated by dormant endospores gaining access to the mammalian host and becoming engulfed by regional macrophages (Mf). During systemic anthrax, late stage events include vegetative growth in the blood to very high titers and the release of the anthrax toxin complex, which causes disease symptoms and death. This study focuses on the early events occurring during the first few hours of the *B. anthracis* infectious cycle, from endospore germination up to and including release of the vegetative cell from phagocytes. We found that newly vegetative bacilli escaped from the phagocytic vesicles of cultured Mf and replicated within the cytoplasm of these cells. Release from the Mf occurred 4-6 hours after endospore phagocytosis, timing that correlates with anthrax infection of test animals. Genetic analysis from this study indicates that the toxin plasmid pXO1 is required for release from the Mf, while the capsule plasmid pXO2 is not. The transactivator atxA, located on pXO1, was also found to be essential for release, but the toxin genes themselves were not required. This suggests that Mf-release of anthrax bacilli, like its other known virulence factors, may be atxA-regulated. Further tests that are presented in this study suggest that the putative escape gene is phosphatidycholine-specific phospholipase C (PC-PLC).

Ecology and Epidemiology Poster Session II – P1

Monday, 5:30–6:30 p.m. | McDowell Hall

Board 2B. Orally Administered Live Spore Vaccines Fail To Induce Immunity against Anthrax

P. TURNBULL[1], K. FOWLER[2], L. BAILLIE[3]

[1]Arjemptur Technology Ltd, Salisbury, UNITED KINGDOM; [2]Centre for Applied Microbiology & Research, Porton Down, UNITED KINGDOM; [3]DERA CBD, Porton Down, UNITED KINGDOM

Guinea pigs were fed by stomach tube on days 0, 21, and 42 with 1 ml volumes of spore suspensions of *B. anthracis* Sterne strain 34F2, *B. subtilis* WB600 pPA 101-1 and B. globigii (control group), all pre-adjusted to 10E8 cfu per ml. Fecal samples were collected from each individual animal before each feeding session for IgA anti-spore or anti-PA antibody analysis. For six days after each feeding session, all feces were collected for enumeration of excreted spores. On day 64, after blood samples had been taken, the guinea pigs were challenged intramuscularly with 10E3 cfu of *B. anthracis* Vollum strain spores. In all three groups the administered organisms were found exclusively in the spore form in the feces and became undetectable by day six. There was no evidence of multiplication within the gastrointestinal tracts of the animals. In the *B. anthracis* group, total numbers detected in the feces were within one to two logs of the numbers fed. In the case of the recombinant *B. subtilis* and B. globigii, the numbers detected in the faeces were two to three logs below the numbers fed but these species differences may simply reflect the problems of identifying the *B. subtilis* and B. globigii colonies within a background of other faecal flora. Increasingly rapid declines in fecal counts, reflecting accumulating immunity, were not readily apparent in successive feeding sessions and IgG and IgA anti-spore or anti-PA antibodies were not detected in fecal and/or serum samples. All the guinea pigs succumbed to the challenge with no significant differences in time to death between the livestock Sterne strain vaccine group, the recombinant *B. subtilis* group and B. globigii controls. The indications, therefore, are that the vaccine strains failed to colonize or produce PA and thereby induce immunity. (This work was carried out at Centre for Applied Microbiology & Research, Porton Down, UK)

Board 3B. Characterization of the Variable-number Tandem Repeats in *vrrA* from *Bacillus anthracis* Isolates in Korea

Y. M. PARK, W. K. SEONG, J. S. HAN, H. B. OH

Laboratory of Bacterial toxins, Department of Microbiology, National Institute of Health, Eunpyung-gu, Seoul,122-701, REPUBLIC OF KOREA

Bacillus anthracis is known to be one of the most genetically homogeneous pathogens, making strain discrimination particularly difficult. In an effort to genetic characterization of *B. anthracis* prevailed in Korea, we investigated 46 isolates from anthrax outbreaks and environmental samples. Three isolates were from the outbreak of gastrointestinal anthrax in 1994, 12 isolates were from the outbreak of cutaneous anthrax in 2000, and 31 isolates were from the environmental samples collected around the several pastures in Korea from 1997 to 2000. Thirty-eight strains were virulent and 8 strains were avirulent. The strains of *B. anthracis* were identified by multiplex PCR for the detection of *pag*, *cap*, *Ba813* gene. As a result of VNTR analysis, 46 isolates of *B. anthracis* revealed 3 polymorphism of two to four copies of the 12-bp tandem repeat 5¡¯-CAATATCAACAA-3¡¯. The GPR-1/GPR-2 primers were used in PCR to amplified 1.1 kb DNA fragments of *B. anthracis* chromosomal DNA from strains containing the different VNTR sequences. As a result of DNA sequencing of VNTR amplicon using GPR-4/GPR-5 primer, *B. anthracis* ATCC 14578 and 23 isolates were shown the same $VNTR_2$ category. *B. anthracis* ATCC 14185, 14186, Sterne and the other 14 isolates fell into $VNTR_4$ category. The remaining 12 isolates all from the outbreak in Changnyung showed as the $VNTR_3$.

Board 4B. Re-emergence of Anthrax in Kazakhstan

D. A. ASHFORD[1], C. WOODS[1], K. OSPANOV[2]

[1]CDC, Atlanta, GA; [2]Kazakhstan Ministry of Health, Almaty, KAZAKHSTAN

Human anthrax was reported infrequently in the former Soviet Union state of Kazakhstan (10%-15% of 230 annual Soviet Union cases between 1983 and 1989). The incidence remained low after the country became independent in 1991. However, during 1997 and 1998, several outbreaks of cutaneous disease in humans were identified. We reviewed Kazakhstan's anthrax surveillance for 1958-1998 and current livestock ownership and vaccination practices. To evaluate risk factors, we used human anthrax outbreaks as sentinels to identify a cohort of persons who had contact with anthrax-infected animals. A case was defined as a person with a lesion consistent with cutaneous anthrax or laboratory-confirmed systemic anthrax. Between 1958 and 1998, 4,214 persons with anthrax were reported in Kazakhstan. Incidence peaked in 1966 (2.4/100,000) and declined to an average of 0.13/100,000 (range 0.05-0.19) between 1991 and 1996. In 1997, human anthrax outbreaks resulted in a threefold increase in incidence (0.42/100,000) compared with the preceding 5 years. Between 1991 and 1997, the percentage of privately owned livestock increased from 15% to 80%, and vaccination of livestock decreased from 100% to 30%. We identified seven outbreaks, five in 1997 and two in 1998, in which 53 of 309 human contacts of infected animals developed anthrax. The median age was 35 (range 5-71); 31(58%) were male. All 53 survived; 51(96%) had cutaneous anthrax, with 82% of lesions occurring on the hand or arm, and 2 had gastrointestinal disease. All source animals were privately owned and unvaccinated. Slaughtering (RR 2.9; 95% CI 1.2-6.9) and butchering (RR 3.1; 95% CI 1.3-7.4) infected animals were independently associated with disease. Reduction in anthrax vaccination may be responsible for the outbreaks of 1997-98. Control programs should include expanded animal vaccination and public education regarding meat safety.

Board 7A. Human Anthrax Associated with a North Dakota Epizootic: What's the Risk?

P. M. DULL[1], T. GOMEZ[2], M. BAJANI[1], J. JOHNSEN[1], T. CLEMENTS[3], G. LUDWIG[3], L. WHITE[4], E. HALVORSON[4], L. SCHULER[5], L. SHIRELEY[6], D. ASHFORD[1]

[1]CDC, Atlanta, GA; [2]USDA, APHIS, Atlanta, GA; [3]USAMRIID, Fort Detrick, MD; [4]USDA, APHIS, Bismarck, ND; [5]Department of Agriculture, Bismarck, ND; [6]Department of Health, Bismarck, ND

Background: Anthrax, a bacterial zoonosis caused by *Bacillus anthracis*, may be transmitted to humans from infected animals. The last reported case of human anthrax in the United States occurred in 1992. On September 20, 2000, CDC was notified of a suspect human anthrax case by the North Dakota Department of Health. Initial investigation revealed that the suspect case occurred during a larger anthrax epizootic in unvaccinated livestock. We investigated this suspect case, the epizootic, and assessed the risk of human anthrax. Methods: We administered a questionnaire to livestock owners or ranch foremen at 32 sites of confirmed livestock-anthrax cases. Information was obtained regarding animal populations and deaths, vaccination status, environmental conditions, management of affected carcasses, management of remaining herd, and evidence of associated human disease. Serologic analysis (protective antigen(PA) antibody) was performed on 35 of 91 exposed individuals (26/79 farm workers, 5/6 veterinarians, 4/6 laboratory personnel). Also, we interviewed the suspected case-patient and collected sera for PA antibody testing. Results: Between July 13 and August 26, 2000, an anthrax epizootic in six counties in eastern North Dakota resulted in the death of 127 animals (108 cattle, 9 horses and 10 bison) on 36 pastures. Death rates were highest in bulls (36.4%) and horses (8.6%). The outbreak occurred after a period of very heavy rain. On August 28, 2000, a 67-year-old farmer presented with a painless lesion on his face consistent with cutaneous anthrax; he was treated with ciprofloxacin and recovered. Serologic testing was positive (1:200) for PA antibodies. His exposure involved gloved manipulation of the head and hooves of several anthrax-infected cattle carcasses. Between July 6 and September 24, 6 veterinarians and 39 farm-workers handled 127 anthrax-infected animal carcasses in 6 counties with 7 veterinarians and 7600 farm-workers. Of those from whom exposure histories were obtained, all the veterinarians (6/6) and 33% (13/39) of the farm-workers wore gloves. No other symptomatic human anthrax was discovered. Conclusions: As a result of a re-emergence of anthrax among livestock in North Dakota, one case of human anthrax occurred among 39 (2.6%) persons handling infected animals during the largest epizootic in North Dakota history. Forty of 7600 (0.5%) farm-workers and 6 of 7 (90%) veterinarians in this 6-county region were at potential risk of anthrax. The risk for human disease can be minimized by appropriate management of carcasses, timely vaccination and/or treatment of remaining herd and proper personal protective

equipment. Early diagnosis and treatment are essential to management of cutaneous anthrax disease, and physicians in the affected parts of the U.S. should be alerted to the potential for human anthrax among those working with livestock.

Board 9A. The Kameido Incident: Documentation of a Failed Bioterrorist Attack

H. TAKAHASHI[1], A. F. KAUFMANN[2], K. L. SMITH[3], P. KEIM[3], K. TANIGUCHI[1,4]

[1]Infectious Disease Surveillance Center and Department of Pathology, National Institute of Infectious Diseases, Tokyo, JAPAN; [2]Consultant, Stone Mountain, GA; [3]Northern Arizona University, Flagstaff, AZ; [4]World Health Organization, Geneva, SWITZERLAND

In 1993, a liquid suspension of *Bacillus anthracis* was sprayed from the roof of an 8 story building in Kameido, a locale in metropolitan Tokyo, Japan. The Aum Shinrikyo, a religious doomsday cult, conducted the attack as part of their efforts to trigger an apocalyptic global nuclear war. The spray was intermittently generated from one of two large dispersal devices over a roughly 24 hour period on July 1 and 2. Nearby residents complained of a foul odor associated with the spraying. A gelatin-like fluid, which fell on the side of the building, was collected in test tubes. During a 1996 arraignment of Aum Shinrikyo members, the nature of the attempted attack was first made public. The agent used was identified as a vaccine strain of *B. anthracis*. A retrospective telephone survey of area medical facilities revealed no illnesses compatible with inhalation, cutaneous, or intestinal anthrax. In 1999, culture of the gelatin-like fluid collected from the building exterior revealed spores of *B. anthracis* in a viable concentration of 103 CFU/ml. The Kameido strain was found to be pX01 positive and pX02 negative. Multiple-locus variable-number tandem repeat analysis (MLVA) found marker differences between the Kameido strain and over 700 previously MLVA genotyped strains, including the Pasteur vaccine strain, the Sterne vaccine strain and the Russian STI-1 vaccine strain. The closest genotype was a strain isolated from China that differed by 2 marker alleles. The attack apparently failed due to the non-encapsulated strain used, or other contributing factors such as an inefficient spray device and low agent concentration in the dispersed fluid.

Classification, Identification, and Detection
Poster Session II – P2

Monday, 5:30–6:30 p.m. | McDowell Hall

Board 11A. Outer Spore Coat Gene Molecular Genetic Diversity of The *Bacillus anthracis* Group

T. LEIGHTON, **E. R. EL-HELOW**, D. XIA, Y. GOMEZ

University of California, Berkeley, CA

Members of the *B. anthracis* group are environmentally ubiquitous and include closely related infectious agents of insects and mammals. They include *B. anthracis*, which causes anthrax in animals and man, *B. cereus*, which causes human food poisoning, *B. thuringiensis*, which is an insect pathogen and is used as a biological insecticide, and *B. mycoides*, which is considered a harmless saprophyte. Due to the lack of specific markers for the *B. anthracis* host chromosome, molecular diagnosis of anthrax has relied on detection of plasmid virulence genes. This situation is unsatisfactory, since positive identification of the anthrax chromosome, in addition to virulence plasmid signatures, is required to differentiate non-pathogenic, vaccine, and fully virulent strains of anthrax. Similar concerns apply to the environmental analysis of *B. thuringiensis* strains that have been classified previously based on flagellar antigen serology or the presence of insecticidal cry plasmids. Ecological theory predicts that certain protein-coding genes could have the appropriate resolving power for discrimination among near-neighbor species. We have demonstrated previously that sspE signatures (279-288bp) can distinguish *B. anthracis* from all of the near-neighbor species analyzed and reveal new taxonomic fine structure within the *B. anthracis* group. In this study, DNA sequence analysis of an outer spore coat gene (cotK) coding region (150 bp) allowed a phylogenetic separation of 36 genetically diverse *B. anthracis* group strains into 23 nucleic acid (genotypes) and 12 protein sequence clusters (proteotypes). Computational analysis of upstream cotK regulatory regions (approximately 106 bp) clustered the strains studied into 30 different genotypes. These results suggest that upstream regulatory sequences may diverge at a more rapid rate than coding sequences. All of the *B. anthracis* group cotK genes have a near canonical Shine-Dalgarno ribosome binding site sequence GAGGTG, utilize an ATG translation start codon, and terminate translation with a TAA stop codon. A comparison of sspE and cotK sequence clustering suggests that spore structural constituents are promising tools for investigating the molecular evolution of the *B. anthracis* group. The genetic diversity identified among cotK genes may be particularly useful for the development of specific antibody reagents for the detection of spore surfaces and vaccines that would protect against aerosol exposure to anthrax spores.

Board 13A. Multi-Locus Sequence Typing (MLST) of *Bacillus anthracis* and Its Close Relatives

R. T. OKINAKA[1], R. SVENSSON[1], C. HELMA[1], K. HILL[1], P. S. WHITE[1], P. KEIM[2], P. J. JACKSON[1]

[1]Los Alamos National Laboratory, Los Alamos, NM; [2]Northern Arizona University, Flagstaff, AZ

Multi-Locus Sequence Typing (MLST) combines the polymerase chain reaction (PCR) and DNA sequencing to gather precise sequence information from multiple regions of the genomes of bacteria and other organisms. The method targets the analysis of common regions in related species and is therefore similar to ribosomal gene (rDNA) analysis that has been used to establish the phylogenetic relationships among organisms residing in the microbial world. We have used MLST to expand these analyses to multiple regions that evolve more quickly than rDNA to build larger data sets that increase genetic resolution, particularly for close relatives that have little or no variation in their ribosomal DNA sequence. We report the comparison of sequences from at least five loci in multiple strains of *Bacillus anthracis* and approximately 100 *Bacillus cereus/Bacillus thuringiensis* isolates. There are very few differences in the sequences from 25 isolates of *B. anthracis* but more than 300 single nucleotide polymorphisms (SNP) have been identified in comparisons between the concensus *B. anthracis* sequences and the first 50 *B. cereus/B. thuringiensis* isolates that have been analyzed. These data sets allow *B.*

anthracis to be clearly distinguished at the genomic level from all of its closest relatives. The phylogenetic tree derived from these data are remarkably similar to the more global analysis represented by the amplified fragment length polymorphism (AFLP) technique (See P. Jackson et al., these proceedings).

Board 15A. Fluorescent Amplified Fragment Length Polymorphism (AFLP) Analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacilllus thuringiensis* Isolates

K. K. HILL, L. O. TICKNOR, A. RICHARDSON, P. E. PARDINGTON, P. J. JACKSON

Los Alamos National Laboratory, Los Alamos, NM

DNA isolated from over 300 isolates of *Bacillus thuringiensis*, *Bacillus cereus*, and *Bacillus anthracis* was analyzed by fluorescent Amplified Fragment Length Polymorphism (AFLP). The *B. thuringiensis* and *B. cereus* isolates were from diverse sources and geographic locations including soil isolates from Norway, global isolates from dairy and rice products, type strains from the American Type Culture Collection, and over 200 *B. thuringiensis* strains representing over 25 different serovars from the USDA collection. Twenty-four isolates of *B. anthracis* were also included. The phylogenetic tree generated from the AFLP data revealed the extensive diversity of *B. thuringiensis* and *B. cereus* compared to the monomorphic nature of *B. anthracis*. All of the *B. anthracis* isolates were more closely related to each other, while the many *B. cereus* and *B. thuringiensis* isolates populated the entire tree. At least five distinct branches were defined and each branch included isolates of *B. cereus* interspersed with *B. thuringiensis*. One of the five branches included all of the *B. anthracis* isolates along with many virulent or infectious *B. cereus* and *B. thuringiensis* isolates. *B. thuringiensis* kurstaki (ATCC 33679) mapped well away from *B. anthracis* and the virulent *B. cereus* isolates. Many of the *B. thuringiensis* isolates within a particular serovar were more closely related to each other than to other *B. thuringiensis* isolates. The interspersion of *B. cereus* and *B. thuringiensis* within this tree supports the recent arguments that these should be considered to be a single species. However, the presence of five branches of the phylogenetic tree also suggests that these two species might also be reclassified as multiple species. It is clear from of these results that all *B. anthracis* isolates are distinct from all *B. cereus* and *B. thuringiensis* isolates so far analyzed.

Board 17A. Mutation Rate Estimates for VNTR Regions in *Bacillus anthracis*

G. ZINSER, D. L. SOLOMON, S. MILLER, M. BAI, J. E. FARLOW, J. FLINT, M. FLEMING, P. KEIM

Northern Arizona University, Flagstaff, AZ

Genetic diversity in the highly monomorphic bacterium, *B. anthracis*, can be detected by analyzing variable regions in the genome called Variable Number Tandem Repeats (VNTRs). The mutation rates of 36 VNTRs were determined using the Sterne (pX01+, pXO2-) strain of *Bacillus anthracis* using a parallel serial passage experimental design. One colony of *B. anthracis* was subcultured into 423 separate lines and passaged through forty-three transfers, resulting in approximately five hundred thousand effective generations. DNA was extracted from the final colony of each line and then used to support amplification of the regions. Mutational events in these regions were detected by measuring amplicon size differences by fluorescent gel electrophoresis on an ABI 377 sequencer. The mutation rates were then calculated by dividing the number of mutation events per VNTR by the number of generations. Most of the VNTR regions exhibited no mutations and, hence, had rate estimates of less than 6X10E-6 mutations per generation (one event observation in this experiment). Several loci have multiple mutations, with fastest rate estimates being >1X10E-5. Both insertions and deletion mutations were observed. In addition to single repeat mutations, we also observed double repeat changes at a lower frequency. VNTR mutation rates estimates will allow us to understand the divergence among strains in both an epidemiological and phylogenetic setting.

Board 18B. Evolutionary Relationships in *Bacillus anthracis* Based Upon MLVA

G. ZINSER, **D. L. SOLOMON**, L. B. PRICE, A. M. KLEVYTSKA, K. L. SMITH, J. M. SCHUPP, P. KEIM

Northern Arizona University, Flagstaff, AZ

Until recently, it has been difficult to describe evolutionary relationships between relatively monomorphic *Bacillus anthracis* isolates from around the world. To overcome this problem, genetic markers were developed based on variable regions in the *B. anthracis* genome, through a system called multi-locus VNTR analysis (MLVA). In order to increase the information provided by the MLVA system, 28 additional polymorphic markers were developed using two distinct methods. The first method involved identifying variable amplified fragment length polymorphism (AFLP) markers, sequencing these markers, and then generating flanking primers for PCR amplification. The second method involved scanning the *B. anthracis* genome for potential variable number tandem repeats (VNTRs) using DNA sequence analysis software. Primers were then designed which flanked promising VNTRs. The resulting primer sets from both methods were tested for variability against a set of 88 *B. anthracis* isolates previously shown through MLVA to have distinct genotypes. Phylogenetic analyses with the new markers were then performed on the 88 isolates. UPGMA cluster analysis, as well as maximum parsimony analysis revealed very similar relationships to what were seen with the original MLVA system. For example, the previously known A, B1 and B2 groups remain as distinct clusters. The development of these additional markers serves to strengthen the previous MLVA system by confirming evolutionary relationships.

Board 20A. Rifampicin Resistance in *Bacillus anthracis* and *B. cereus*

S. FINE-PERCY, C. TIPTON, **A. VOGLER**, J. BUSCH, P. KEIM

Northern Arizona University, Flagstaff, AZ

The antibiotic rifampicin kills bacteria by binding to and inhibiting RNA polymerase. Resistance to rifampicin is commonly due to mutations in the beta subunit of RNA polymerase, encoded by the rpoB gene. These mutations result in a change in the rifampicin-binding site. Our goal has been to characterize the rpoB gene sequence from both wild type and rif-resistant mutants in *B. cereus* and *B. anthracis*. Rifampicin resistant mutants of *B. cereus* and attentuated *B. anthracis* (Ames: pXO1-, pXO2-) were produced using UV light and selection using high concentrations of rifampicin. PCR and DNA sequencing primers were designed using *B. subtilis* and Staphylococcus aureus gene sequences. Complete gene

sequences have been generated for *B. cereus* and *B. anthracis*. The known rifampicin-binding region of the rpoB gene was sequenced from the resistant mutants and compared to wild type genes to identify changes. Twenty-five *B. cereus* and twenty-five *B. anthracis* mutants have been isolated and the rpoB gene has been sequenced from most of these mutants. Mutations have been characterized as to type and clustering of mutations. In addition, we have characterized 20 *B. anthracis* spontaneous Rif resistant mutants. The mutations that have been identified were clustered in a specific region of rpoB consistent with studies from other bacteria. A Luria-Delbruck experimental design has been used to measure the mutation rate that leads to Rif resistance in *B. anthracis*. While rif resistance has been long known in these bacteria, we now know the molecular basis for this.

Board 21A. VrrC: A hypervariable DNA translocase from *B. anthracis*

A. KLEVYSTKA, J. M. SCHUPP, D. ADAIR, P. S. KEIM

Northern Arizona University, Flagstaff, AZ

Molecular variation among different strains of *Bacillus anthracis* is vanishingly small. The majority of the examples of variation are associated with variable number tandem repeats (VNTRs), some of which create "in frame" mutations in proteins. The vrrC locus is an extreme example as there are three VNTRs present in the 5' portion of the structural genic region. Variation in these regions is currently independent, though sequence similarities suggest a common evolutionary origin. It seems probable that the three VNTRs have evolved from a single ancestral VNTR. Eight haplotypes have been detected for the vrrC1 region, four haplotypes have been observed for the vrrC2 region, and to date, three haplotypes have been noted for the vrrC3 region. Combined, these regions account for nearly 1500 nucleotides and 500 amino acids encoded by the vrrC gene of most strains. The predicted protein product of this locus shows very high similarity to YtpT and SpoIIIE in *Bacillus* subtilis and FtsK in *Eschericia coli*. The strongest similarity is in the 3' portion of the gene as there is less conservation among taxa and genes in the 5' region. It is the 5' genic region that also contains the VNTRs. Comparable VNTRs are not observed in the *E. coli* and *B. subtilis* genes. The SpoIIIE and FtsK have been shown to function as DNA translocases during sporulation and replication, respectively. The role of the vrrC VNTRs in a DNA translocase is unknown, but many different alleles are present in *B. anthracis*, which potentially could provide variation for evolutionary adaptation.

Board 23B. Development of Methods for Extracting DNA from Bacterial Spores

T. TOROK[1], T. LEIGHTON[2], J. HUNTER-CEVERA[3]

[1]Lawrence Berkeley National Laboratory, Berkeley, CA; [2]University of California Berkeley, Berkeley, CA; [3]University of Maryland Biotechnology Institute, , MD

Historically the application of molecular biology to the analysis of microbial community structure and activity was meant to overcome existing classical microbiological methodological limitations. Two primary beneficiaries of this development have been microbial diversity and forensics. Under real world forensic scenarios, one may be dealing with a mixture of spores or spores that have been damaged, aged (weathered), or otherwise altered physically. Therefore, the initial requirement is to extract sufficient quantity of DNA from spores contained in real-world sample matrices to ensure reliable DNA amplification and subsequent identification of specific assay signatures. In this project Lawrence Berkeley National Laboratory scientists addressed the FBI Laboratory's requirements for the development of improved methods, and procedures for processing samples, which are suspected of containing hazardous biological materials, including biological warfare agents. A critical state-of-the-art review of the literature highlighted the shortcomings and the needs and gaps of the available information. It became apparent that researchers investigating entire microbial communities in environmental samples either ignore the presence of spores in their samples or are simply not aware that their techniques may disrupt only a fraction or none of the bacterial endospores present. Recent development in DNA extraction did not make the isolation and quantification of amplifiable DNA from bacterial endospores any less trivial. Alternative immunosorbent spore ELISA assays, immunomagnetic spore detection approaches, and in situ amplification or whole cell hybridization though attractive have been compromised by environmental sample matrices, lack of specificity, and difficulties in achieving spore permeabilization. Thus, there is still a pressing need for spore DNA-based analysis techniques. Therefore, our goal was to design and validate pure spore preparation protocols and methods for sample preparation, spore disruption, and DNA extraction, recovery and quantitation. Using the developed techniques PCR-amplifiable DNA was extracted from a variety of bacterial endospores. Also, these methods were adapted, improved, and/or modified for the analysis of spiked samples.

Board 25B. Multiple-locus Variable-number Tandem Repeat Analysis, as a Tool To Study Genetic Relationship within French *Bacillus anthracis* Strains.

A. FOUET[1], K. SMITH[2], M. LÉVY[1], C. KEYS[2], M. MOCK[1], J. VAISSAIRE[3], **P. KEIM**[2]

[1]Institut Pasteur, Paris, FRANCE; [2]Northern Arizona University, Flagstaff, AZ; [3]AFSSA, Maison-Alfort, FRANCE

Outbreaks of anthrax zoonose still regularly occurs in France. *Bacillus anthracis* is the causal agent of anthrax, a serious often fatal infection in both livestock and humans. Animals are infected by contact with soilborne spores. Humans are infected only incidently, via contact with diseased animals, or their waste products. Virulent strains of *B. anthracis* produce toxins and are encapsulated. These strains have two virulence plasmids: pXO1 and pXO2, encoding the toxins and the capsule synthetic activity, respectively. *B. anthracis* is one of the most monomorphic pathogenic bacterium described. Numerous studies have shown that techniques used to discriminate between strains in other species have low discriminatory power within the *B. anthracis* strains. Recently, we have developed a multi-locus VNTR analysis (MLVA) for the molecular typing of *B. anthracis* strains (1). Short nucleotide sequences that are repeated multiple times vary in copy number, creating length polymorphisms. We defined 6 such fragments on the chromosome and 1 on each of the plasmids. The MLVA technique was then applied on different strain collections, including the French one which contains samples that have been collected during the last 15 years in various parts of France. Non-virulent *Bacillus* soil strains, found in fields where anthrax had occured, were found to be close relatives to *B. anthracis* (2).

They were also included in this study, as well as a *Bacillus thuringiensis* strain, isolated from a human wound. The French *B. anthracis* strains belong to different genotype groups. Moreover, some strains were shown to define groups not previously described among the 89 found through a worldwide survey comprising 419 strains. Finally, the French *B. anthracis* strains were assigned to the two defined clusters which could suggest diverse geographical origins. 1) Keim P., et al., 2000, J. Bact., 182, 2928-2936. 2) Patra G., et al., 1998, J. Clin. Micro., 36, 3412-3414.

Board 27B. Siderophore Production in *Bacillus anthracis* and Related Organisms

J. E. L. ARCENEAUX, B. L. GARNER, **B. R. BYERS**

Univ. Mississippi Med. Ctr., Jackson, MS

Presumptive production of a siderophore (iron transport agent) by the Sterne strain of *Bacillus anthracis* was evaluated on CAS siderophore detection agar. When colonies were patched onto CAS agar, excretion of a possible siderophore by *B. anthracis* was observed as a typical siderophore halo around the growth after 24-36 hour incubation (either 37 C or 30 C). The CAS agar reactions of *Bacillus cereus*, *Bacillus thuringiensis* and a known siderophore (schizokinen) producer, *Bacillus megaterium*, also were tested. When incubated at both 37 C and 30 C, *B. megaterium* produced more schizokinen at 37 C, while *B. thuringiensis* was siderophore positive at its optimum of 30 C. At both temperatures, *B. cereus* displayed only small white to blue-green, atypical halos which may indicate lack of siderophore production by this organism. The iron chelating phenolate 3,4-dihydroxybenzoic acid (protocatechuic acid) was identified (by ethyl acetate extraction and thin layer chromatography) in culture filtrates of *B. anthracis* grown in low iron (0.1 μM) liquid medium, confirming an earlier report (Koehler, Pasha and Williams, Abstr. 92 Genl. Mtg. Amer. Soc. Microbiol.,1992, 46). However, this phenolate was not the apparent *B. anthracis* siderophore since it failed to give the appropriate CAS reaction. Some of the iron acquisition capabilities of *B. anthracis* were determined by inhibition of growth with the chelating agent ethylene-di(*o*-hydroxyphenyl- acetic acid) (EDDA) and its reversal with various iron sources. The minimal growth inhibitory concentration of EDDA was high (2 mg/ml), indicating the presence of an efficient iron acquisition system possibly involving the suspected siderophore. EDDA inhibition was reversed by iron salts, heme, iron-transferrin, and the siderophores Desferal and mycobactin J, but not by iron-lactoferrin, suggesting multiple, specific iron uptake mechanisms in *B. anthracis*. Purification and identification studies of the *B. anthracis* siderophore are in progress.

Board 29B. Validation of the Use of Gamma Phage for Identifying *Bacillus anthracis*

T. G. ABSHIRE[1], J. E. BROWN[2], J. D. TESKA[1], C. M. ALLAN[3], S. L. REDUS[3], J. W. EZZELL[1]

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In 1999, the CDC/APHL Laboratory Response Network to Bioterrorism selected the gamma phage lysis of *Bacillus anthracis* as a specific method for identifying the bacterium. Brown and Cherry originally isolated the gamma phage in the 1950s and showed it to specifically lyse vegetative *Bacillus anthracis*. That property has been used as the basis for a specific identification assay on culture plates at USAMRIID since the mid-1980s. We report here our results of a study to validate the assay for routine use. The primary intent of the assay is to test non-hemolytic, ground-glass appearing bacterial colonies appearing from culture of clinical or non-clinical samples on 5% sheep blood agar. Specifically, this assay was designed to demonstrate clear or partially clear circular zones of lysis on a bacterial lawn at the site of gamma phage inoculation following incubation at 35 ± 2 C for 24 hours. The validation study tested the assay for specificity, precision, and ruggedness. When tested with 50 *B. anthracis* strains and 50 similar non-anthracis *Bacillus* species using whole cell fatty acid analysis (MIDI, Inc., Newark, DE) for identification confirmation of each test strain, analytical specificity was >95%. We confirmed that several rare *B. anthracis* strains are refractory to the gamma phage as tested and that two non-anthracis strains, *B. cereus* ATCC 4342 and *B. mycoides* CDC 680 are susceptible. Repeatability, day-to-day precision, and analyst-to-analyst precision were superior. The assay is rugged to variation between phage lots, reduced phage activity, variable amounts of bacterial inoculation and incubation times (as short as 8 hours). The data suggests that the assay is satisfactory for routine use.

Cell Structure and Function Poster Session II – P3

Monday, 5:30–6:30 p.m. | McDowell Hall

Board 31B. Analysis of *Bacillus anthracis* Spore Surface Antigens with Monoclonal Antibodies

P. SYLVESTRE[1,2], E. TOSI-COUTURE[1], M. MOCK[1]

[1]Institut Pasteur, Paris cedex 15, FRANCE; [2]Centre d'etudes du Bouchet, Vert-Le-Petit, FRANCE

Monoclonal antibodies were raised against *Bacillus anthacis* spores by immunization of mice with formalized spores of attenuated Sterne strain or of a virulent wild type strain. In each case numerous monoclonal antibodies were obtained. As shown by indirect immunofluorescence (IF) tests, they reacted specifically with *B. anthracis* spores and did not cross react with vegetative forms. However, these antibodies cross reacted with spores of other closely related *Bacillus* species. Immunoblotting analysis on extracts of whole spore and exosporium fragments showed that several antibodies were directed against an epitope present in an antigen of a molecular mass higher than 250 KDa. This antigen was shown to be a glycoprotein. Its location in the outer regions of the exosporium was confirmed by immunogold electron microscopy.

An other fusion was obtained after immunization of mice with native exosporium fragments from spores of the Sterne strain. These monoclonal antibodies were tested by indirect immunofluorescence tests with spores of several strains of *B. anthracis* and of other *Bacillus* species. Some antibodies were found to recognize exclusively *B. anthracis* spores. However, Western blot analysis revealed that all antibodies reacted with epitopes of the 250 KDa antigen. These results indicate that the 250 KDa antigen presents several epitopes. One or more are commun with spores of related *Bacillus* species, whereas at least one epitope seems to be restricted to *B. anthracis*. The monoclonal antibodies directed against this epitope may help for the differenciation of *B. anthracis* from non-anthrax *Bacillus*.

Board 33B. A Developmentally Regulated S-layer Switch in *Bacillus anthracis*

T. MIGNOT, S. MESNAGE, E. TOSI-COUTURE, M. MOCK, A. FOUET

Institut Pasteur, Paris cedex 15, FRANCE

Bacillus anthracis synthesizes two S-layer proteins : Sap and EA1 encoded by the clustered chromosomal genes *sap* and *eag*. Previous studies have shown that both proteins are present simultaneously at the cell surface and are anchored via a conserved N-terminal S-Layer Homology (SLH) domain. Both S-layer proteins are each able to form an S-layer network under the capsule. This suggested that two distinct lattices may coexist at the surface, or, Sap and EA1 could be subunits of a more complex network.

We investigated the regulation of *B. anthracis* S-layer genes using genetic and biochemical approaches. We showed that *sap* and *eag* are transcribed from independent transcriptional units as stable mRNas. Transcriptional fusions using the *lacZ* reporter gene revealed that *sap* is expressed in the exponential phase whereas *eag* is expressed in thestationary phase. The exponential phase repression of *eag* was shown to be dependent upon the synthesis of Sap. As demonstrated by gel mobility shift DNA binding assays, Sap has the ability to bind specifically on the *eag* promoter. Therefore, Sap acts as a direct repressor of *eag* in exponential phase. Sap is both a morphogenetic protein and a transcription factor.

As shown by protein quantification experiments and immunoelectron microscopy, the differential expression of *sap* and *eag* leads to the progressive inversion of the Sap/EA1 ratio at the surface during the stationary phase. Moreover, EA1 appears at the surface by mediating the release of Sap into the culture medium. This is not due to competition at the sites of protein anchoring, it is more probably the result of an EA1 network spreading accross the surface. Our results suggest that *B. anthracis* sequentially harbors two mutually exclusive S-layers as it develops.

Board 34A. The Development and Use of scFv antibody Fragments To Characterise the Vegetative Phase S-Layer Protein EA1 of Various *Bacillus* Species

G. K. FRITH, H. UPPINGTON, C. REDMOND, L. FULOP, S. HIBBS, J. DEWEY

Antibody Detection Group, CBDE, Wiltshire, UNITED KINGDOM

There is a need to be able to characterise structural differences between closely related *Bacillus* species. Unfortunately, there are limited immunlogical reagents available to characterise such differences. We will describe the generation and production of single chain Fv (scFv) antibody fragments, using phage display technology, that recognise one such vegetative phase-associated protein, EA1, isolated from *B.anthracis*. We have been able to demonstrate the scFv recognises purified EA1, vegetative cell lysates, inactivated spores and soluble spore lysates. We are currently attempting to characterise the epitopes on the EA1 protein recognised by these scFv's. Further we hope to use these scFv's to characterise the EA1 proteins from closely related *Bacillus* species.

Board 36B. The Development and Use of scFv Antibody Fragments To Characterise *Bacillus anthracis* Spore Surface Antigens.

G. FRITH, H. UPPINGTON, C. REDMOND, L. FULOP, S. HIBBS, J.DEWEY

Antibody Detection Group, CBDE, Wiltshire, UNITED KINGDOM

We have generated a number of single chain Fv (scFv) antibody fragments, derived from 3 independent hybridoma cell lines, using a novel phage display and selection method. The hybridomas were originally derived from mice immunised with irradiated ***Bacillus anthracis*** spores.

Currently, we are using the scFv's to characterise differences between spore preparations of other *B. anthracis* strains and several closely related Bacillus species. We are investigating the use of scFv proteins on various real-time detection surfaces, and, how scFv bioactivity and orientation affect the efficiency of detection.

We are attempting to use the scFv's to immunoaffinity purify individual spore proteins with the intention of sequencing the purified product. This may allow us a route to recombinant expression of individual *B.anthracis* spore antigens.

Board 38B. Assessment of *Bacillus anthracis* Growth using Quantitative "Real-time" PCR

A. A. FADL, M. E. YOUNG, H. TAEGTMEYER, T. M. KOEHLER

University of Texas-Houston Health Sciences Center, Houston, TX

In inhalation anthrax, *Bacillus anthracis* spores germinate in alveolar macrophages and become metabolically active vegetative cells. Microscopic studies indicate that the bacteria can proliferate inside macrophages before either losing viability or being released from the host cell. In order to examine the physiology of *B. anthracis* inside macrophages, intracellular growth of the bacterium must be assessed. Accurate quantitation of vegetative *B. anthracis* cells inside macrophages is not easy. Within a given population, *B. anthracis* cells grow in chains of varying length. Thus, colony-forming units can represent one or multiple cells. Microscopic determination of cell numbers can be inaccurate because septa are often difficult to discern. Moreover, the number of fields and samples that must be examined to acquire significant data make manual counting prohibitively time-consuming. We have developed a rapid and quantitative method employing the Roche LightCycler and quantitative "real time" PCR to determine the copy-number of a specific DNA template in populations of *B. anthracis* cells. Forward and reverse primers amplify a conserved region near the *B. anthracis* vrrA gene. The PCR is performed in the presence of an oligonucleotide probe that hybridizes to a sequence within the amplicon. The probe is labeled at the 5' end with a reporter fluorochrome and at the 3' end with a quencher fluorochrome. During each PCR cycle, as Taq polymerase synthesizes a copy of the DNA template, it displaces and cleaves the probe with a Taq-associated 5'-3' exonuclease activity. During each round of PCR amplification, one molecule of probe is cleaved per molecule of template and there is a corresponding increase in fluorescence. The amount of probe cleaved is directly proportional to the amount of template DNA. Amounts of template DNA in unknown samples can be determined from a standard curve generated using known amounts of a synthetic amplicon. When vegetative cells from a pure culture of *B.*

anthracis were used in the reaction, the amount of target DNA detected correlated with the optical density of the culture. Preliminary results using *B. anthracis* - infected RAW 264.7 macrophages indicate that this method can be used to assess changes in the number of *B. anthracis* DNA template molecules in a population of infected macrophages over time. We anticipate use of this system as a DNA-based method for rapid and quantitative measurement of *B. anthracis* growth.

Genomics and Gene Regulation

Tuesday, 8:30 a.m.–10:30 a.m. | Key Auditorium

The *Bacillus anthracis* Genome Project

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Whole-genome sequencing of a *Bacillus anthracis* Ames isolate (pXO1- pXO2-) is nearing completion. The initial phase of the project, random sequencing of small- and large- insert libraries has been completed and efforts are being directed currently to closing gaps between assemblies. Methods for generation sequence information to link contigs include direct genome-walking, combinatorial multiplex PCR and multiplex inverse PCR.

Many portions of the *B. anthracis* sequence appear to have similar gene content and organization to the archetypal non-pathogenic *B. subtilis* and to the recently sequenced *B. halodurans* genome. At least 60% of *B. anthracis* ORFs have homologues to known *B. subtilis* genes. These include many spore-coat and spore-germination determinants believed to play in important role in virulence. There are many genes without homologues in *B. subtilis* that could be important in anthrax infection, including several hemolysins and phospholipase genes. Also notable was the presence in the genome of numerous copies of a conserved 16 bp palidrome known to be a target of the *B. thuriengiensis* positive regulator of extracellular virulence determinants, PlcR. However, the *B. anthracis plcR* gene contains a potential loss-of-function deletion.

The pXO plasmids that contain the key virulence genes encoding toxin and capsule have recently been sequenced. Although the plasmids appear to have undergone frequent rearrangements, there are few apparent instances of gene transfer between plasmid and chromosome, suggesting possible recent arrival of the episome into *B. anthracis*.

The *B. anthracis* genome data will provide vital information on evolution and pathogenicity determinants that will enhance efforts to design countermeasures against this potent biowarfare threat. To further exploit the genome sequence data we are planning a number of functional genomic studies including the contruction of a DNA gene microarray at TIGR.

B. anthracis sequence data can be accessed through the TIGR microbial database site www.tirg.org/tdb/mdb/mdb.html.

Global Gene Expression and Specificity of *B. anthracis* pX01 ORFs

J. PANNUCCI, E. WILLIAMS, R. CARY, P. PARDINGTON, R. OKINAKA, C. R. KUSKE

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The *B. anthracis* large virulence plasmid, pX01 (181.7 kb), encodes at least 143 ORFs. Putative functions could be assigned to only 35 of the ORFs by sequence similarity (Okinaka et al. J. Bacteriology, 1999) and little is known about the function of the remaining majority of the pXO1 plasmid sequence. To determine which of the predicted pXO1 plasmid ORFs are expressed, RNA was isolated from *B. anthracis* Sterne cells grown under different temperature and carbonate conditions, including the conditions that induce expression of the toxin genes. Gene expression of pX01 ORFs was analyzed by fluorescence-based microarray hybridization of reverse transcribed total RNA. Results to date indicate there are pX01 genes being expressed in addition to the toxin genes; some in a culture condition dependent manner. We also sought to identify regions of pXO1 sequence that are conserved among other *Bacillus* spp., as well as more distantly related bacteria. Using dot blot hybridization and PCR amplification assays, we examined 8 *Bacillus* species and 4 other bacteria for presence of 101 novel pXO1 ORF sequences. The presence of pX01 ORF sequences in other species may indicate conservation of function. Evaluation of conserved ORF sequences can also identify, by process of elimination, pX01 genes that are potentially unique to *B. anthracis*. Four groups of pXO1 ORFs were identified; a small group of ORFs that are conserved across several bacterial genera, a larger group of ORFs with significant sequence similarity to two particular *Bacillus* strains, a small group of ORFs that appear to be unique to *B. anthracis*, and a few pX01 sequences that appeared sporadically among the species tested. Pulsed-field gel electrophoresis revealed large potential plasmid bands present in a *B. cereus* (341kb) and a *B. thuringiensis* (327 kb) strain that hybridized with pX01 DNA probes.

Control of the *Bacillus anthracis* Toxin Genes by the Transition State Regulator *abrB*

E. SAILE, T. M. KOEHLER

University of Texas Houston Health Science Center Medical School, Houston, TX

Bacillus anthracis produces the anthrax toxin proteins in a growth phase-dependent manner when cultured in liquid medium. In a Sterne strain of *B. anthracis*, expression of the toxin genes *pagA, lef,* and *cya* peaks in late log phase and steady state levels of PA, LF, and EF are highest during the transition into stationary phase. Here we show that an apparent transition state regulator differentially affects toxin gene expression. We identified two orthologues of the *B. subtilis* transition state regulator *abrB* in the *B. anthracis* genome: one on the chromosome and one on pXO1. The orthologue located on the chromosome is predicted to encode a 94-amino acid protein that is 85% identical to *B. subtilis* AbrB. The hypothetical protein encoded by ORF105 on pXO1 is 41% identical to *B. subtilis* AbrB but missing 25 amino acids from the amino terminus when compared to the *B. subtilis* protein. Deletion of the pX01-encoded *abrB* orthologue did not affect toxin gene expression. However, a *B. anthracis abrB* null-mutant in which the chromosomal gene was deleted produced PA earlier and in higher amounts than the parent strain. Expression of a

transcriptional *pagA-lacZ* fusion in the *abrB* mutant increased up to 20-fold during early exponential growth compared to the parent strain, and peaked in mid-exponential rather than late exponential phase. When introduced *in trans*, the *B. anthracis abrB* gene complemented the *abrB* null-mutant. In contrast to the strong effect of *abrB* on *pagA* expression, *lef-lacZ* and *cya-lacZ* expression during early log phase growth was increased only two- to threefold in the *abrB* null-mutant. Western hybridization analysis showed only slightly raised LF and EF levels in the mutant. The *abrB* effect on toxin gene expression may be due in part to altered expression of the toxin gene activator *atxA*. Expression of a transcriptional *atxA-lacZ* fusion in the *abrB* null-mutant was two- to threefold higher than in the parent strain. This is the first example of virulence gene regulation by a transition state regulator. Our finding ties *atxA* and the expression of anthrax toxin genes into the complex network of postexponential phase adaptive responses that have been well studied in *B. subtilis*. We believe that AbrB, whose cellular concentration in *B. subtilis* is controlled by the flow of phosphate to Spo0A, determines the timing of toxin gene expression in *B. anthracis*. We hypothesize that the timing of virulence gene expression is important in *B. anthracis* pathogenesis and are now in a position to test the *abrB* mutant for macrophage cytolysis and virulence in the mouse model.

An Evolutionarily Selected Nonsense Mutation in *plc*R Led to the Inactivation of a Complete Regulon in *Bacillus anthracis*

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Bacillus anthracis (*Ba*), *Bacillus thuringiensis* (*Bt*) and *Bacillus cereus* (*Bc*) are members of the *Bacillus cereus* group. These organisms have different pathogenic properties: *Ba* is a mammal pathogen, *Bt* is an insect pathogen and *Bc* has been involved in opportunistic food poisoning. The host specificities of these organisms are mainly conferred by virulence plasmids encoding specific virulence factors, namely, the toxins and the capsule of *Ba* and the Cry proteins of *Bt*. However, *Bt* and *Bc* secrete non specific virulence factors such as enterotoxins, haemolysins and phospholipases. Such enzymatic activities have not been described for *Ba* and its distinction from the other group members has often relied on the basis that it is non haemolytic.

Recently, the synthesis of *Bc* and *Bt* enterotoxins and degradative enzymes has been shown to be controlled by a pleiotropic regulator, PlcR. Interestingly, in *Ba*, *plc*R was found to harbor a nonsense mutation leading to the synthesis of a shorter inactive regulator. These results suggested that the non-haemolytic phenotype of *Ba* could reflect the non-functionality of the *plc*R gene.

In this study we investigated the effect of *plcR* expression in *Ba*. Therefore, *Bt* PlcR was expressed in *Ba* under the control of its own promoter on a multicopy plasmid. *Ba* became haemolytic when an active PlcR was expressed. Moreover, zymogram analysis showed that active protease(s) and phospholipase(s) were induced. Genomic data from TIGR revealed that over 40 genes are putatively under the control of PlcR (T. Read, personal communication). By RT-PCR, we assayed the expression of 4 putative PlcR target genes. The transcription of genes encoding proteins analogous to phospholipase C, perfringolysin O, subtilisin and an SLH-containing surface protein was silent in *Ba* unless PlcR was expressed.

To investigate the role of the PlcR regulon in anthrax pathogenesis, *plc*R was integrated as a single copy on the chromosome of a $pXO1^+$ $pXO2^-$ strain. The recombinant strain displayed the same phenotypes as the strain expressing PlcR from a multicopy plasmid; therefore it will be used in LD50 assays in the mouse infection model.

Taken together our results show that, in *Ba*, the silencing of a complete regulon occured because of a single mutation in a transcriptional activator. This mutation is responsible for the lack of secretion of many degradative enzymes such as haemolysin or lecithinase by *Ba*. Moreover, analysis of the chromosome sequences controlled by PlcR suggests that evolution counter selected a wide panel of functions in *Ba*.

Silent b-lactamase Genes in *Bacillus anthracis*

Y. CHEN, J. SUCCI, T. M. KOEHLER

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Sensitivity to penicillin and other β-lactam antibiotics is a characteristic trait of *Bacillus anthracis* and such antibiotics are recommended for treatment of anthrax. Naturally-occurring penicillin-resistant *B. anthracis* strains have been reported, but these appear to be extremely rare. The frequency of spontaneous resistance to penicillin in the Sterne strain of *B. anthracis* is less than 10^{-8}. Bacterial resistance to β-lactam antibiotics is often associated with synthesis of β-lactamases which inactivate the antibiotic. Here we report the presence of two apparent β-lactamase (*bla*) genes in the penicillin-sensitive Sterne strain of *B. anthracis*. We used different approaches to find the *bla* genes. First, we selected for functional *bla* genes by cloning random fragments of *B. anthracis* chromosomal DNA into *E. coli* and selecting for ampicillin-resistant clones. *B. anthracis* DNA was digested with a variety of restriction enzymes and ligated into plasmid pACYC184. The ligation mixtures were transformed into *E. coli* TG1 and clones were selected on medium containing ampicillin. One ampicillin-resistant transformant was obtained. This clone carried a 5.5-kb *EcoRI* fragment from *B. anthracis*. Sequence analysis revealed that the cloned DNA contained a 927-nt ORF predicted to encode a protein with 93.8 % identity to the type I b-lactamase gene (*bla1*) of *B. cereus*. The ORF was subcloned into the bifunctional vector pAT29 and the resulting plasmid conferred ampicillin-resistance to *E. coli* and *B. subtilis*. These results indicate that the *B. anthracis bla1* gene encodes a functional protein, but the gene is not expressed in *B. anthracis*. A second apparent *bla* gene was found by searching the unfinished TIGR *B. anthracis* genome database for ORFs predicted to encode b-lactamases. We found a partial ORF predicted to encode a protein that is 92.9 % identical to the C-terminal end of the type II β-lactamase of *B. cereus*. DNA adjacent to the 5' end of this *B. anthracis* ORF was cloned using inverse PCR. Further characterization of these two *bla* genes is in progress. We hypothesize that expression of these genes requires some factor not present in *B. anthracis*.

Special Presentation

Tuesday, 11:00 a.m.–12:00 noon | Key Auditorium

The Discovery of the Anthrax Toxin

HARRY SMITH

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Anthrax kills many animal species. It was used to prove Koch's Postulates in 1876. Soon after that the classical bacterial toxins were produced *in vitro* but, up to 1950, a lethal toxin had not been demonstrated in either anthrax bacilli or filtrates from laboratory cultures. The cause of death had been an enigma for seventy years. During the 1950's, a toxin was recognized by examining bacteria and their products obtained directly from guinea pigs dying of anthrax. The toxin was in their plasma and was shown to contain two components. It was then produced *in vitro* and a third component recognized. The work rekindled interest in bacterial toxins after a period of dormancy and showed, for the first time, that toxins could be multicomponent. It also demonstrated that new knowledge about the determinants of bacterial pathogenicity could be obtained by examining organisms grown *in vivo.*

Molecular Interactions of Anthrax Proteins

Tuesday, 2:00 p.m.–4:00 p.m. | Key Auditorium

Dominant Negative Mutants of Protective Antigen: An Approach to Therapy of Anthrax

B. R. SELLMAN, M. MOUREZ, R. COLLIER

Harvard Medical School, Boston, MA

The protective antigen component (PA) of anthrax toxin binds to the cell surface and is proteolytically cleaved. The 63 kDa receptor-bound C-terminal fragment of PA oligomerizes into a ring-like heptamer and binds edema factor (EF) and lethal factor (LF). Upon exposure to low pH in the endosome, PA undergoes a conformational change resulting in the translocation of EF or LF across the bounding membrane. We have identified mutants of PA that bind cells, assemble into the heptamer and bind EF and LF but are translocation deficient. When mixed with wild-type PA (WT-PA), several of the translocation deficient mutants exhibit a dominant negative (DN) phenotype. The DN-PAs inhibit the ability of WT-PA to deliver of a toxic ligand into the cytosol by assembling into an inactive hetero-heptameric complex with WT-PA. When mixed with a lethal dose of WT-PA + LF, the DN-PAs protected Fisher 344 rats, even at sub-stoichiometric amounts. Additionally, the DN-PAs elicited a protective immune response similar to that of WT-PA. DN-PAs represent a unique approach to therapy for a microbial disease and could prove useful as a combined therapeutic/vaccine for clinical anthrax

Crystal Structure of the Anthrax Toxin Lethal Factor

ROBERT LIDDINGTON

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In collaboration with the groups of John Collier and Steven Leppla, we have determined the crystal structure of the Lethal Factor at a resolution of 2.2 Å. Lethal Factor is a 90 kDa protein consisting of four domains: Domain 1, which is sufficient for binding Protective Antigen, shows unexpectedly high homology to Domain 4. However, the region which would be occupied by the active site is mutated so that Zn coordination is impossible. Domain 2 bears a striking resemblance to the ADP-ribosylating toxin of B. cereus, although the active site residues have been mutated; the NAD binding groove remains and may have been recruited to provide an extended specificity pocket for the MAPKK substrate. Domain 3 is a helical bundle that is inserted into Domain 2; from its location it is likely to restrict access to the active site of potential substrates such as the loops of a globular protein; that is, it may contribute towards specificity for a terminal "tail" of a protein substrate. Domain 4, the C-terminal domain, contains the metalloprotease site; the most similar structure in the DALI data base is the Zn metalloprotease, thermolysin. Although the overall fold of the domain is only distantly related, the zinc coordination sites superimpose well, suggesting that that the Lethal Factor catalytic domain is a classic zinc metalloprotease. A model for the interaction of Lethal Factor with the MAPKK N-terminus will be presented.

Crystallographic and Biochemical Studies of the Edema Factor Toxin from *Bacillus anthracis*

C. L. DRUM[1], W. TANG[2], A. BOHM[3]

[1]BBRI / University of Chicago, Watertown, MA; [2]University of Chicago, Chicago, IL; [3]Boston Biomedical Research Institute, Watertown, MA

Bacillus anthracis secretes three exotoxins, protective antigen, lethal factor and edema factor. Both lethal factor and edema factor can be functionally divided into two parts, a protective antigen binding region and a region containing catalytic activity. The catalytic portion of edema factor is a 60kD adenylyl cyclase that is activated by binding calmodulin. Here we report the crystal structures of the catalytic part of edema factor alone and in complex with calmodulin. This part of the enzyme is roughly composed of three globular domains, with the active site located in a cleft between domains one and two. As predicted from proteolytic digestion experiments, the C-terminal domain of edema factor makes an extensive interaction with calmodulin and undergoes a large conformational change upon calmodulin binding. Calmodulin is shown to adopt an extended conformation when complexed to edema factor. This represents a novel interaction mechanism since previously solved structures of calmodulin in complex with peptides from other calmodulin-effectors show both heads of calmodulin collapsed around the peptide target. Previous biochemical data indicating that calcium binding within calmodulin's C-terminal head is more important than that of the N-terminal head is also interpreted within the framework of the molecular model. No

direct contacts are seen between calmodulin and the active site. A comparison of complexed and enzyme alone structures implies a structural mechanism for catalytic activation. In spite of virtually undetectable sequence homology, analysis of the catalytic cleft suggests a di-metal mediated cyclization reaction similar to that of the mammalian adenylyl cyclases.

Drum, CL, et al. (2000) An Extended Conformation of Calmodulin Induces Interactions Between the Structural Domains of Adenylyl Cyclase from *Bacillus anthracis* to Promote Catalysis. J. Biol. Chem. 275:36334-36340

Responses of the Macrophage to Lethal Toxin

MAHTAB MOAYERI

National Institute of Dental and Craniofacial Research, NIH

The lethal toxin (LT) produced by *Bacillus anthracis* is the major virulence factor responsible for the pathogenesis of anthrax. Primary macrophages and macrophage cell lines derived from sensitive mice (M^S) are killed by LT over 90-120 minutes. Resistant macrophages and cell lines (M^R) are not killed by toxin. A range of protective agents, many of which are antioxidants, can prevent LT-mediated cytotoxicity. In order to better understand the cellular parameters and responses which define resistance to and protection against toxin-mediated death, we investigated early cellular events in response to LT treatment. Mitochondrial membrane potential changes in susceptible cells were monitored with the JC-9 dye, using confocal microscopy and flow cytometry. Sudden loss of membrane potential was not an early event in toxin-treated cells and occurred only at 60-70 minutes. Cleavage of known intracellular targets Mek1, Mek2 and Mek3 began at 30 minutes in M^S and M^R as well as under a wide range of antioxidant and non-antioxidant protective conditions. This cleavage proceeded with similar kinetics in all tested cell types and conditions and appears unrelated to resistance or protection. Activation of stress response transcription factors such as NFkB in response to toxin treatment was observed post-60 minutes using a secreted alkaline phosphatase (SEAP) reporter system, and electrophoretic mobility shift assays (EMSAs). Our efforts did not identify any events unique to M^S prior to 60 minutes. In order to screen a large number of potential "early" (pre-60 minute) gene expression changes in toxin-treated macrophage lines, Clontech Atlas™ mouse gene expression arrays were utilized to detect up or down-regulated genes in LT treated BMAJ (M^R) and RAW 264.7 (M^S) cells. A family of co-regulated genes controlled by ARE (antioxidant response element) and Nrf-2 transcription factor responsive to LT have been identified by these methods and are presented here. The involvement of specific cellular components in resistance to LT is discussed.

Genomics and Gene Regulation
Poster Session III – P4

Tuesday, 4:30 p.m.–5:30 p.m. | McDowell Hall

Board 23A. Construction and Evaluation of a System for Directed Signature-Tagged Mutagenesis (DSTM) of Chromosomal and Extrachromosomal Genes of *Bacillus anthracis*

I. MENDELSON[1], S. COHEN[1], E. MAMROUD[1], T. CHITLARU[1], N. ARIEL[1], H. GROSFELD[1], A. M. FRIEDLANDER[2], A. SHAFFERMAN[1]

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A system for *Bacillus anthracis* gene disruption through directed signature-tagged mutagenesis was developed, based on a vector designated pDSTM. The latter is a temperature-sensitive-replication suicide plasmid containing genetic elements for selection and unique cloning sites for PCR amplified tagged-DNA targets. To evaluate the efficiency of the system, the *B. anthracis* chromosomal gene (*glu*) coding for oligo-1,6-glucosidase was targeted by a pDSTM derivative carrying either a 500bp-*glu* PCR amplified tagged DNA fragment or a DNA fragment, carrying a kanamycin resistance cassette, bounded by 500bp-*glu* DNA sequences. Analysis of resulting *glu* mutants revealed that disruption of the *glu* gene occurred through single as well as double crossover recombination events. The *glu* mutants generated by single crossover recombination were genetically stable. The potential of this system for generation of directed signature-tagged mutants in the multicopy virulence pXO1 plasmid, which is known to be replication temperature sensitive, was also evaluated. A 500bp-*pagA* PCR amplified-tagged DNA fragment was cloned into pDSTM and used to transform an Ames strain. Propagation of the *B. anthracis* pDSTM-*pagA* transformants at restrictive temperature and under antibiotic selection yielded the expected pXO1::pDSTM-*pagA* mutant at reasonable frequencies. The pDSTM system described here would be useful in generation of libraries of tagged *B. anthracis* mutants disrupted in selected chromosomal and extrachromosomal (pXO1 or pXO2) genes.

Board 25A. Analysis of *Bacillus anthracis* Genes Encoding Putative Peptidyl Prolyl Isomerases

R. C. WILLIAMS[1], L. BAILLIE[2], C. R. HARWOOD[3], P. T. EMMERSON[1]

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The ability of bacteria to secrete proteins across the cytoplasmic membrane is essential for both the maintenance of viability and, in the case of pathogenic organisms, for virulence. The majority of secretory proteins of Gram-positive bacteria are translocated *via* the Sec-dependent translocase, an important requirement of which is for the proteins to be maintained in an

unfolded state. As they emerge on the *trans* side of the cytoplasmic membrane, secretory proteins must fold rapidly into their native configuration to avoid degradation by "quality control" proteases. Consequently, Gram-positive bacteria have developed a variety of strategies to improve the efficiency with which their secretory proteins are folded into the native configuration. The Sec-dependent pathway of *Bacillus subtilis* has been well-characterised in recent years and we have used this as a basis of a bioinformatical analysis of the incomplete genome sequence of *Bacillus anthracis* to identify putative components of the Sec-dependent pathway. We will report the outcome of this analysis.

PrsA is a key extracytoplasmic folding factor amongst Gram-positive bacteria. PrsA, a putative a peptidyl prolyl isomerase, is a lipoprotein anchored to the outer leaflet of the cytoplasmic membrane where it is thought to facilitate protein folding by catalysing the *cis/trans* isomerization of prolyl residues in secretory proteins. PrsA is essential to the growth of *B. subtilis*, presumably because it is required for the folding of an enzyme or protein involved in cell wall growth or assembly. We have shown that PrsA is required for the secretion of recombinant *B. anthracis* protective antigen (rPA) from *B. subtilis* and overexpression of PrsA leads to higher yields of rPA. In contrast, YacD, a homologue of PrsA appears to have little or no influence on the accumulation of rPA. Bioinformatical analyses of *B. anthracis* have identified three putative homologues of *B. subtilis prsA*. We will present data on the activities of these homologues in rPA producing *B. subtilis* strains which over- and under-express *prsA*.

Board 27A. Identification of Additional Vaccine Candidates in *B. anthracis*

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Bacillus anthracis possesses two well known and well characterised virulence factors, a tripartite exotoxin and an anti-phagocytic capsule; encoded by plasmids pX01 and pX02 respectively. In addition the bacterium expresses other, plasmid and chromosomally encoded genes which contribute to the overall pathogenesis of the organism. Candidate virulence factors include chromosomally encoded extracellular proteases, phospholipases such as cerolysin and S layer proteins. This work aims to identify virulence factors encoded on the *B. anthracis* chromosome which could be incorporated into new generation anthrax vaccines. *B.anthracis* is closely related to *B.cereus*, *B.thuringiensis* and *B.subtilis*, organisms that produce a range of virulence factors, which include extracellular proteases, heamolysins, enterotoxins and phospholipases. Utilising computer based systems we have analysed the *B. anthracis* genome (http://www.tigr.org), for homologues to these genes. Initial database searches (http://genolist.pasteur.fr/Subtilist/) have identified a gene with over 90% homology to nprE (the major neutral protease of *B. subtilis*). The entire gene including the propeptide, mature peptide and signal sequence was sequenced and analysed in a number of *B. anthracis* strains. Treatment of the extracellular supernant of *B. anthracis* cultures with various protease inhibitors confirmed that the majority of proteolytic activity is contributed by a metalloprotease. Analysis of the *B. anthracis* genome has also revealed homologues to both diarrhoeal and emetic enterotoxin genes from *B. cereus*. Portions of the *hlbA, bcet* and *nhe* genes have been amplified and sequenced in a number of *B. anthracis* strains. Phenotypic assays were also carried out to determine whether these genes where functional in *B. anthracis*.

Board 29A. Anthrax Toxin Gene Expression is Dependent upon *sigH*, a Gene Encoding an Alternative Sigma Factor of RNA Polymerase

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University of Texas-Houston Health Science Center, Houston, TX

Expression of the structural genes for the anthrax toxin proteins, *cya* (edema factor), *lef* (lethal factor), and *pagA* (protective antigen), is coordinately controlled by host-related signals and *trans*-acting regulatory genes. Optimal expression of the toxin genes occurs when *Bacillus anthracis* is grown at 37°C in a defined medium containing bicarbonate. Toxin synthesis is highest during late-exponential phase. The toxin genes are located noncontiguously within a 30-kb region of the 182-kb virulence plasmid pXO1. Two pXO1-encoded *trans*-acting regulatory genes have been reported: *atxA* positively affects transcription, while *pagR* is a weak repressor. No regulatory proteins or specific transcription factors have been reported to interact directly with the DNA sequences near the toxin gene promoters. We have determined that the *B. anthracis* homologue of the *Bacillus subtilis sigH* gene, encoding an alternative sigma factor, is required for optimal expression of the toxin genes. *sigH*-dependent genes of *B. subtilis* include a number of genes induced at the end of exponential growth and during initiation of sporulation. The *B. anthracis sigH* gene was found by searching the unfinished TIGR *B. anthracis* genome database. The gene was predicted to encode a protein with 81.6% identity to the SigH of *Bacillus subtilis*. We constructed a *B. anthracis sigH*-null mutant by replacing the gene with an antibiotic resistance gene. The *B. anthracis* mutant was unable to sporulate. Toxin gene expression by the *sigH* mutant was assessed by measuring the β-galactosidase activity of toxin gene promoter-*lacZ* transcriptional fusions and by western hybridization analysis of culture supernates. In optimal conditions for toxin gene expression, transcription of the toxin genes in the *sigH* mutant was reduced to less than 4% of the level observed in the parent strain. No toxin proteins were detected in culture supernates of the *sigH* mutant. Toxin synthesis was recovered when the mutant was complemented *in trans* with the wild type gene. Expression of the toxin gene regulator *atxA* was only slightly affected by *sigH*. β -galactosidase activity of a strain harboring an *atxA-lacZ* transcriptional fusion was reduced 2-fold in a *sigH* mutant compared to the parent strain. In *B. subtilis*, the global regulator *spo0A* is required for the maximal expression of *sigH*. In a *B. anthracis spo0A*-null mutant, toxin gene transcription was reduced to 14-25% of that observed in the parent strain and expression of the *atxA* gene was not altered. These data suggest that the anthrax toxin genes are transcribed by RNA polymerase containing SigH.

Molecular Interactions of Anthrax Proteins
Poster Session III – P5

Tuesday, 4:30 p.m.–5:30 p.m. | McDowell Hall

Board 31A. Platelet, the Novel Action Target of Anthrax

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Abstract The lethal toxin of *Bacillus anthracis*, is a binary toxin consisting of protective antigen which acts as a binding domain, and leathal factor which is a metalloprotease that causes hyperinfammatory responses and eventual cell death. Here, an analysis of biologic effects of lethal toxin on platelet function was carried out. The lethal toxin challenged mice (BALB/C) exhibited a severe hemorrhage mediastinitis-like symptom, and dramatically decreased in the levels of platelet number as well as serum thromboxane B2. In vitro, platelet aggregation was shown significantly inhibited after lethal toxin treatment as determined by whole-blood aggregometer. Immunocytometric examination revealed that P-selectin, an activation marker of platelet, was localized in the dense granule and had impaired the ability to traffic into platelet surface, thus disrupt the following activation processes for the connection between the platelets and the endothelial cells. MEK kinase activity but not P38 is likely to be involved in this anti-thrombocytic effect. Further, co-inoculation of lethal toxin and anti-platelet drugs, such as aspirin and indomethacin would significantly increase in the mortality rate of mice as compared to that of each alone. Together, the results strongly indicated that the function of platelet is highly affected by the anthrax lethal toxin .

Board 33A. Involvement of Residues 147VYYEIGK153 in Binding of Lethal Factor to Protective Antigen of *Bacillus anthracis*

R. BHATNAGAR

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Anthrax toxin is a complex of protective antigen (PA, 735amino acids), lethal factor (LF,776amino acids) edema factor (EF,767amino acids). The toxin follows the A-B model of bacterial toxins where PA (the binding B moiety) binds to cell surface receptor and is cleaved by cell surface proteases into PA63 and PA20, while LF and EF (alternate A moiety) compete for binding to PA63.The PA63-LF/EF complex is internalized into the cytosol and causes different pathogenic responses in animals and cultured cells. Amino acid analysis of LF and EF revealed a strong similarity in the residues 1-300.This region of homology could be the site responsible for the high affinity binding to PA. Amino acid analysis of LF and EF using BESTFIT program of GCG reveals a stretch of seven amino acids in both the catalytic moieties. In the present study, each amino acid of this stretch was replaced by alanine at a time using PCR based mutagenesis. The mutagenized PCR products were cloned in expression vector pQE30 having hexa- histidine tag. The mutant proteins were purified using Ni-NTA affinity and anion exchange chromatography. These proteins were checked for their ability to lyse J774A.1 cells and their ability to bind to receptor bound PA/ trypsin nicked PA in solution .Out of seven mutants, Y148, Y149, I151 and K153 mutants were found to be deficient in their ability to lyse J774A.1 cells. Further analysis of mutant proteins, revealed that their binding ability to PA63 was drastically reduced. On the basis of these results, we propose that Y148, Y149, I151 and K153 amino acids play a crucial role in the process of binding of LF to PA63 during intoxication process.

Board 36A. Cell Surface Display of *Bacillus anthracis* Protective Antigen on *Escherichia coli* by Using Ice Nucleation Protein

S. KIM, H. KIM, **Y. CHAI**

Hanyang Univ., Ansan, REPUBLIC OF KOREA

The ice-nucleation protein (Inp) is a glycosyl phosphatidylinositol-anchored outer membrane protein found in some Gram-negative bacteria. Using *Pseudomonas syringae* Inp as an anchoring motif, we investigated the functional display of a foreign protein, *Bacillus anthracis* protective antigen (Pag), on the surface of *Escherichia coli.* The cells expressing Inp-Pag were found to retain both the ice-nucleation enzyme activity, and whole-cell protective antigen activity, indicating the functional expression of Inp-Pag hybrid protein on the cell surface. The surface localization was further verified by immunofluorescence microscopy, Western blotting assay, whole-cell dot blotting, and ice nucleation activity assay. No growth inhibition or changes in the outer membrane integrity were observed upon the induction of fusion protein synthesis. Viability of the cells was also maintained over 48 hours in the stationary phase. We confirmed that the Inp anchoring motif can be stably expressed for foreign protein as large as 83 kDa. When the Inp-Pag displayed cells were used as the whole-cell enzyme-linked immunosorbent assay, Pag was efficiently synthesized from *E. coli.*

Board 38A. Nontoxic Anthrax Toxin Component Is a Unique Delivery System for Protein Presentation

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State Research Center for Applied Microbiology, Obolensk, 142279 Russia

The structural basis of the T cell immune recognition is now well understood. $CD4^+$ and $CD8^+$T cells possess clonally distributed receptors ($\alpha\beta$ TCR), which are specific for the complex of peptides derived from protein antigens and specifically bound to class I or class II MHC molecules. The reason for the existence of the two distinct classes of MHC molecules, both of which possess the peptide binding activity and are recognized by $\alpha\beta$ TCR, appears to relate to different functions of $CD4^+$ and $CD8^+$T cells. $CD4^+$T cells interact productively with B cells, macrophages, and dendritic cells that primarily acquire a multiplicity of antigenic proteins from the external milieu. CD8+T cells or cytotoxic T lymphocytes (CTLs) are specialized for destruction of cells that have been infected or undergone malignant transformation, where the presence of a particular protein marks the cell for death before mature virus, intracellular bacteria or further tissue invasion occurs. For activation CTLs, the presented protein antigen must

be translocated into the cytosol and presented to class I MHC molecules. The macrophages infected with Brucella, can be a good target for CTLs, if CTLs are induced, and TCR complexes recognizing MHC-I molecules, alongside with *Brucella* CTL-peptides, are produced at their surface. In order to activate CTL, we have used a vehicle containing nontoxic components of the Anthrax Toxin: the Protective Antigen (PA) and associative domain of the Lethal Factor (LFn).

The main goal of our investigations is to develop the CTL-component of the molecular vaccine against Brucellosis. We propose two *Brucella* outer membrane proteins (31 kDa and 25 kDa) as the model antigens for these investigations. Brucellosis is still one of the hottest problems of the veterinary world. Brucellosis is a complex infectious disease mainly affecting the immune system of the infected animals; it is classified as an extremely dangerous infection transmitted from ill animals to humans. The recommended vaccine is the live attenuated *Brucella* strain 19. Vaccination with this strain is associated with serious side effects, which are similar to clinical symptoms of Brucellosis itself. The development of a new safe vaccine for the prophylaxis of Brucellosis is a very attractive perspective. In the present investigation, we have decided to study the macrophage-like cells J774A.1 infected with *Brucella* as the target for CTLs induced by the delivery system of the Anthrax toxin conjugated with the main Brucella membrane proteins BCSP31 and omp25.

Murine macrophage-like J774A.1 cells were provided from the All-Russia Bank of Tissue Cultures (St.-Petersburg, Russia). A *B. abortus* vaccine strain and virulent *B. melitensis* strain were obtained from the collection of the "Microbe" Institute (Saratov). CTL-assay was performed with the use of the non-radioactive CytoTox96R kit (Promega). Macrophage-like J774A.1 cells infected with *Brucella* were used as the target cells. J774A.1 cells (10^5) were incubated with *B. abortus* strain 19 cells (10^6) for 1 hour and then washed. After 1/2/3/4 days, the infected cells were used as the target cells for CTLs.

The results obtained allow a conclusion that highly specific CTL can be effectively induced *in vivo* by the nontoxic components of Anthrax Toxin. The growth rate of *Brucella abortus* strain 19 obtained from the organs of vaccinated mice was 20-100 times lower than that from the control mice. It has been demonstrated that the fused proteins can activate T-cell proliferation of splenocytes in the presence of PA. The efficiency of MHC-I presentation of the omp25 protein attains the maximal level within 1 day after the infection of target cells with *Brucella* and decreases by the 4th day. At the same time, MHC-I presentation of BSCP31 is much lower, as compared with the omp25 presentation, but it remains stable over 4 days after the infection of the target cells. It was especially valuable that in our studies, macrophage-like cells infected with virulent *Brucella* were used as target cells. It is also noteworthy that presentation of the omp25 antigen obtained from the virulent *Brucella* was lower, as compared with the vaccine strain 19. At the same time, this presentation was stable throughout the experiment (4 days).

Immunoprophylaxis and Treatment
Poster Session III – P6

Tuesday, 4:30 p.m.–5:30 p.m. | McDowell Hall

Board 1B. Development of a *B. anthracis* Spore Vaccine Expressing Recombinant Protective Antigen

S. COHEN, I. MENDELSON, Z. ALTBOUM, D. KOBILER, E. ELHANANI, T. BINO, M. LEITNER, I. INBAR, H. ROSENBERG, Y. GOZES, R. BARAK, M. FISHER, C. KRONMAN, B. VELAN, **A. SHAFFERMAN**

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Several highly attenuated spore forming nontoxinogenic and nonencapsulated *B. anthracis* vaccines were constructed, differing in levels of expression of recombinant protective antigen (rPA). Biochemical analyses as well as biological and immunological tests demonstrated that the rPA retains all the characteristics of native PA. A single immunization of guinea pigs with $5x10^7$ spores of one of these recombinant strains, MASC-10, expressing high levels of rPA (>100µg/ml) from a constitutive alpha-amylase promoter, induced high titers of neutralizing anti-PA antibodies. This immune response was long lasting, for at least 12 months, and provided protection against a lethal challenge of virulent (Vollum) anthrax spores. The recombinant *B. anthracis* spore vaccine appears to be more efficacious than the vegetative cell vaccine. Furthermore, while results clearly demonstrate a direct correlation between the level of expression of PA and the potency of the vaccine, they suggest also that some *B. anthracis* spore associated antigen(s) may contribute in a significant manner to protective immunity.

Board 4A. Post Exposure Treatment of Guinea pigs Infected with *Bacillus anthracis* Spores.

Z. ALTBOUM, Y. GOZES, A. BARNEA, Y. PAPIR, A. LAZAR, D. KOBILER

Israel Institute for Biological Reseach, Ness-Ziona, ISRAEL

The efficacy of post exposure prophylaxis against *B. anthracis* infection was tested in a guinea pigs model. Animals were infected via the intranasal route with ~ 50 LD50 Vollum spores. Twenty four hours post infection guinea pigs were treated 3 times per day with antibiotics, either alone or in combination with immunization with a PA based vaccine. The efficacy of four antibiotics was tested: cefazolin, trimethoprim - sulfamethoxazole (TMP/SMX), erythromycin and tetracycline. Treatment with cefazolin and TMP/SMX for 14 days failed to protect animals. Treatment with erythromycin for 14 days prevented the death of the infected animals, however, upon cessation of antibiotic administration all animals died from anthrax. Treatment with tetracycline for 14 days protected the animals, yet upon termination of the antibiotic injection only 25% of the animals survived. Prolongation of the treatment with tetracycline to 30 days increased the percentage of the surviving animals to 55%, for at least 30 days post cessation of antibiotic administration. Efficient treatment of Vollum infected animals, was achieved by a combined treatment with tetracycline for 30

days and vaccination with PA vaccine, on days 8 and 22 post infection. Following this combined treatment, animals were observed for additional 30 days. About 90% of the guinea pigs survived and developed antibodies to PA, and all resisted intramuscular challenge with 30 LD50 Vollum spores. These results indicate that treatment with tetracycline can protect *B. anthracis* infected animals, however, the antibiotic treatment *per se*, does not prevent re-establishment of the disease upon treatment termination. A combined treatment with tetracycline and PA vaccine can provide a long-term protection, preventing anthrax disease reoccurrence.

Board 7B. *In vitro* Characterization of the Phagocytosis and Fate of Anthrax Spores in Macrophages and the Effects of Anti-PA Antibody.

S. WELKOS, S. WEEKS, S. LITTLE, I. MENDELSON, A. FRIEDLANDER

USAMRIID, Frederick, MD

Antibodies (Abs) to the protective antigen (PA) component of the anthrax toxin have anti-spore as well as anti-toxin activities. Purified anti-PA Abs and anti-PA antisera enhance the phagocytosis by murine-derived macrophages (MQs) of spores of the Ames and Sterne strains and retard the germination of spores *in vitro*. Using culture medium that supported phagocytosis without stimulating spore germination (Dulbecco's minimal essential medium with 10% horse serum), the fate after phagocytosis of untreated and anti-PA treated spores was further studied. The spores germinated within RAW264.7 MQs, and germination was associated with a rapid decline in spore viability. Exposure of macrophages to inhibitors of phagoendosomal acidification (*i.e.*, bafilomycin A and chloroquine) reduced the efficiency of macrophage killing and allowed outgrowth and replication of the organisms. Treating spores with anti-PA Ab stimulated their phagocytosis and was associated with enhanced MQ killing of the spores. The enhanced killing of spores was correlated with the greater extent of germination of anti-PA-treated spores after phagocytosis. A PA knockout mutant of the Ames strain exhibited none of the effects associated with anti-PA treatment of the parental strain. Thus, the anti-PA Ab-specific immunity induced by vaccines has anti-spore activities. Our goal is to assess its role in impeding the early stages of infection with *B. anthracis* and in promoting protective immunity.

Board 9B. Efficacy of the U.S. Human Anthrax Vaccine in Various Laboratory Animal Models

P. F. FELLOWS, M. K. LINSCOTT, B. E. IVINS, M. M. PITT, C. A. ROSSI, S. F. LITTLE, P. GIBBS, A. M. FRIEDLANDER

USAMRIID, FREDERICK, MD

The current U.S. human anthrax vaccine, Anthrax Vaccine Adsorbed (AVA), consists of aluminum hydroxide-adsorbed supernatant material, primarily protective antigen (PA), from fermentor cultures of a toxigenic, non-encapsulated strain of *Bacillus anthracis*, V770-NP1-R. Although human cases of anthrax are relatively rare in the United States, several countries throughout the world suffer from endemic outbreaks of the disease. There is also concern about the possible use of *B. anthracis* as a weapon. Therefore, it is important to determine whether there are isolates of the organism for which the vaccine is not efficacious. To clarify the relationship between AVA efficacy, animal models, and isolate diversity we compared the efficacy of AVA in several animal models challenged with diverse *B. anthracis isolates*. Hartley guinea pigs were initially used to screen these isolates. Subsequently, New Zealand White rabbits, rhesus macaques and Golden Syrian hamsters were vaccinated with AVA and challenged with spores from those isolates found to be most virulent in the vaccinated guinea pigs. Vaccination of guinea pigs with AVA provided varying degrees of protection against challenge with virulent strains. Rabbits and rhesus macaques were well protected against anthrax spore challenge by AVA vaccination, whereas hamsters were afforded no protection by AVA. These data may reflect differences in either disease pathogenesis or intrinsic antibody response with respect to the animal model, and they emphasize the importance of examining multiple animal species in an attempt to model the effectiveness of human anthrax vaccine.

Board 11B. Characterisation of the Human Immune Response to the Current UK Anthrax Vaccine

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The current UK human anthrax vaccine has been in use since 1963 and during that time over 50 000 doses have been given. The vaccine consists of the alum-precipitated, cell-free culture supernatant of the Sterne strain of *Bacillus anthracis*. In addition to the key immunogen, protective antigen (PA), the vaccine also contains a number of other bacterial and media-derived proteins which may account for the transient side effects experienced by some individuals. It is surprising, given the history of the use, that so little is known concerning the immunological basis of the immune response induced by this vaccine. In an attempt to better understand the mechanisms involved we have characterized the IgG antibody (ELISA) and T cell population (FACS) responses of vaccinees to a range of *B. anthracis* derived antigens. The highest titres were as expected, generated against PA. The level of anti-PA antibody is likely to be an important indication of the protected status of an individual. Time course studies of Porton staff demonstrated that anti-PA antibody levels peaked two weeks post the annual booster dose but fell back to pre-immunisation levels 10 weeks later. In contrast a separate study amongst Swedish volunteers demonstrated a significant anti-PA titre in 30% of vaccinees 15 months post immunisation. These results suggest that the Heterogeneity of the host plays a role in modulating the PA specific antibody response.

Board 13B. The Expression *Bacillus anthracis* Protective Antigen in *E.coli*

N. WALKER[1], J. MILLER[1], A. TOPPING[2], E. TAYLOR[2], C. REDMOND[1], H. FLICK-SMITH[1], L. BAILLIE[1], E. WILLIAMSON[1]

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Bacillus anthracis, the eitological agent of anthrax, secretes a tripartite toxin comprising of protective antigen (PA), lethal factor and edema factor. PA is the dominant antigen in the current UK vaccine, which consists of an alum-precipitated cell-free filtrate of Sterne strain cultures grown to maximise PA content. We have previously described the production and

protective efficacy of recombinant PA expressed in *Bacillus* subtilis. Here we present data comparing the exisiting *B. subtilis* process with a new process for PA expression in *E.coli* and outline a process for manufacture. The *E.coli* process is highly productive and produces material of high protein purity which is low in residuals such as endotoxin. Product was analysed by SDS-PAGE and Western blotting and the tertiary structure confirmed using circular dichroism. The protective efficacy against a subcutaneous challenge with the STI strain of *B. anthracis* in the murine model will also be reported.

Board 15B. Biochemical Analysis of the UK Licensed Anthrax Vaccine

B. HALLIS, C. QUINN, S. NOONAN, G. LLOYD, M. HUDSON

CAMR, Salisbury, UNITED KINGDOM

The manufacturing process for the UK licensed anthrax vaccine was developed during the immediate post-second world war years. The vaccine was licensed for human use in the UK in the 1960s and used in the prevention of occupationally-acquired anthrax. The bacterial growth parameters and downstream processes were developed to enhance production in vitro of the protective antigens and to minimise reactogenicity by reduction of oedematous and lethal activity. The UK licensed anthrax vaccine is, therefore, a largely undefined mixture of cellular components from culture supernatants of toxigenic, non-capsulated *Bacillus anthracis* strain Sterne 34F2 precipitated in the presence of alum (potassium aluminium sulphate). Although some analysis of the protective components of alum-precipitated 'Stern' strain culture supernatants has been undertaken, the exact composition of the product is not known and the components unquantified. Data will be presented on the biochemical analysis of this vaccine in terms of total protein, carbohydrate and nucleic acid content and functional and immunoreactive anthrax toxin components (protective antigen, lethal factor & oedema factor) and S-layer proteins (Sap & EA-1). A reproducible method for the extraction of alum-precipitated proteins, including the toxin components, was critical to these analyses.

Board 17B. Efficacy of Protection Against Anthrax by the Associative Domain of the Lethal Factor

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All of the currently used live and chemical anthrax vaccines produce or contain, respectively, each of the components of the Anthrax Toxins (Sterne, 1939; Ginzburg, 1943; Leppla, S.H., 1995). Moreover, the protective activity of the lethal factor (LF) component itself has been clearly demonstrated (Ivins, B. et al., 1986; Turnbull, P. *et al.*, 1986; Guidi-Rontani, C. *et al.*, 1999).

In view of this, we studied the protective activity of the fragment of LF devoid of enzymatic activity, *ie.*, the associative domain involved in the binding of LF with the protective antigen. Several preparations were constructed: LF, LFn, LFn-cys, BSCP31-LFn. In addition, dimer LFn-cys-cys-LFn was obtained by means of chemical conjugation at the S-S bond. The preparations were obtained from recombinant *E.coli* strains and purified by affinity chromatography on Ni^{+2} Sepharose.

Mice and virulent *B.anthracis* strain 81/1 (LD_{50} = 1-5 spores) were chosen for biological experiments. The infecting dose was 10 LD_{50}. Mice received two subcutaneous injections (with a 3-week interval) of the preparations with incomplete Freund'adjuvant. Immunized mice were infected with the virulent strain 2 or 3 weeks after the second immunization. Small blood samples were taken from the immunized mice to determine anti-LFn titers by ELISA.

Our experiments have demonstrated that the preparation containing *Brucella* outer membrane protein BSCP31 had the highest level of protective activity (100%). The activity of dimer LFn-cys-cys-LFn was lower (80%), and the activities of monomer LFn and LF were the least (60%). Antibody titers increased with the increasing dose of immunization (linear dependence up to the dose of 5 mg); the response at higher doses, however, was lower. The strong protective activity of the preparation containing BSCP31 is probably due to the induction of gamma-interferon synthesis by this protein (unpublished data). Injection of preparations with PA (at doses below 500 ng) considerably increased anti-LF titers and, correspondingly, the rate of protection. These results agree with the immunomodulating role suggested for PA (Brossier, F. *et al.*, 2000).

Board 20B. Recombinant Expression of Anthrax Toxin Components in a Non-toxigenic *Bacillus anthracis*

C. QUINN, N. SILMAN, S. NOONAN, B. HALLIS, A. ROBERTS, M. HUDSON, G. LLOYD

CAMR, Salisbury, UNITED KINGDOM

Anthrax continues to be a problem both in the case of naturally and occupationally acquired infections by *Bacillus anthracis*. Oedema Factor [EF], Lethal Factor [LF] & Protective Antigen [PA] are the three anthrax toxin antigens which are involved in both infections and are also the main constituents of the anthrax vaccine. Novel expression vectors for production of the three anthrax toxin components were constructed. The non-toxigenic host (*Bacillus anthracis* UM23C1-1) was employed for expression; this has the advantages of using native expression systems and also that high levels of expression were achieved without the problems associated with proteolytic cleavage, commonly observed with alternative expression hosts. This novel system has allowed the production of each individual toxin component, which can be readily purified and retain functional and antigenic properties. The new expression vectors utilise fusion proteins at the N-terminus to aid purification. The system chosen employs the synthetic IgG binding domain (ZZ-domain). Initial vectors constructs were modified substantially to overcome issues of stability and transformation efficiency. These modifications will be presented and discussed in detail. The expressed proteins have been shown to be functional and immunogenic by in vitro assays. These reagents have been successfully applied to the development of diagnostic assays and are key to the characterisation of anthrax vaccines.

Board 39A. Comparison of AVA Relative Potency Results Calculated Using Time of Survival to those Calculated Using Survivors Alone

R. MYERS[1], C. BOTEZAN[2], P. ANDREW[2], B. KINTNER[2], C. SHIH[2], L. GIRI[2], S. RASTOGI[2]

[1]BioPort Corporation, Lansing, MI; [2]BioPort Corporation, Lansing

In the "vaccination/challenge" test model, a vaccine's potency with respect to a reference vaccine is often evaluated by the number of survivors at the end of the test. In contrast, the licensed potency test for Anthrax Vaccine Adsorbed (AVA) takes into account not only the survivors but also the time of survival for animals succumbing during the test period. In this paper we evaluate whether the inclusion of time of survival in the calculation of relative potency is a refinement over calculations utilizing number of survivors alone, without regard to time. In a technical study consisting of 11 replicate tests of *B. anthracis* vaccination/challenge, reference vaccine was compared against itself. AVA Lot FAV032 was used both as reference vaccine as well as test vaccine. In each test, four dilutions (1:1.6, 1:4, 1:10, and 1:25) of the reference and test vaccines were used. A set of 12 BioPort guinea pigs (GP), 6 males and 6 females, each weighing 315 – 385 g on the day of the vaccination was assigned to each of the four dilutions of reference and test vaccines. On the 14th day post vaccination, GPs were challenged with 1000 *B. anthracis* spores and deaths were recorded daily for a 10-day observation period. The numbers of surviving GPs at each dilution and each vaccine were recorded at the end of test. We also calculated the number of days each GP survived over the 10-day observation period. From the number of surviving GPs at the end of the test, we calculated by the probit method the relative potency of the test vaccine with respect to the reference vaccine. From the numbers of days GPs survived at each dilution, we calculated the mean number of days GPs survived (MNDS) by dilution for each vaccine. The best linear regression lines $Y = A + B.X$ for reference and test were calculated, where Y = MNDS and X = log (dil). Using parallel line approach the relative potencies for the 11 replicate tests were calculated. The two sets of relative potencies are compared. It is concluded that the application of the information on the length of survival in calculating relative potencies provides more precise estimates of RP than using only the number of survivors at the end of the observation period. Moreover, there were significantly less non-graded responses with the length of survival approach versus number of survivors alone.

Board 40A. Elimination of Gender Effect Afforded by Relative Potency Test Procedure in Evaluation of Anthrax Vaccine Adsorbed (AVA)

S. RASTOGI[1], C. BOTEZAN[2], A. PHIPPS[2], B. KINTNER[2], C. SHIH[2], L. GIRI[2], R. MYERS[2]

[1]SR Consultants, Inc., N. Potomac, MD; [2]BioPort Corporation, Lansing

It is our experience as well as that of United States Army Medical Research Institute for Infectious Diseases and Centre for Applied Microbiology Research that the AVA vaccinated male guinea pigs (GP) succumb to lethal challenge of *B. anthracis* spores in greater number than similarly vaccinated and challenged female GPs. However, we strongly believe that the relative potency (RP) test procedure eliminates the effect of gender on the potency results, as it does for many other factors. To confirm this hypothesis, we present in this paper the results of our analysis of male and female GP data of a technical study performed at the BioPort. Briefly, in a technical study consisting of 11 replicate tests of *B. anthracis* vaccination/challenge, reference vaccine was compared against itself. AVA Lot FAV032 was used both as reference vaccine as well as test vaccine. In each test, four dilutions (1:1.6, 1:4, 1:10, and 1:25) of the reference and test vaccines were used. A set of 12 BioPort guinea pigs (GP), 6 males and 6 females, each weighing 315 – 385 g on the day of the vaccination was assigned to each of the four dilutions of reference and test vaccines. On the 14th day post vaccination, GPs were challenged with 1000 *B. anthracis* spores and deaths were recorded daily for a 10-day observation period. The numbers of surviving GPs at each dilution and each vaccine were recorded at the end of test. We also calculated the number of days each GP survived over the 10-day observation period. The results were separated by gender. In this presentation it is shown that significantly more female GPs survive compared to the similarly vaccinated and challenged male GPs. The treatments (reference and test vaccines) were not significantly different (obvious, since both vaccines were FAV032). Dilutions were also highly significantly different (this is also obvious). From the number of surviving GPs at the end of the test, we calculated by the probit method the relative potency of the test vaccine with respect to the reference vaccine for each gender. From the numbers of days GPs survived at each dilution, we calculated the mean number of days GPs survived (MNDS) by dilution for each vaccine for each gender. The best linear regression lines $Y = A + B.X$ for reference and test were calculated, where Y = MNDS and X = log (dil). Using parallel line approach the relative potencies for the 11 replicate tests were calculated for each gender. These analyses show that though vaccinated male and female GPs have differential survivals, the relative potency test procedure eliminates the effect of gender on the relative potency results.

Board 41A. Characterization of the US-Licensed Anthrax Vaccine

L. K. WINBERRY, L. BONDOC, S. PARK, L. SIMON, C. N. SHIH, L. GIRI

BioPort Corporation, Lansing, MI

The sole US-licensed anthrax vaccine (Anthrax Vaccine Adsorbed), licensed in 1970 and manufactured by BioPort Corporation in Lansing, Michigan, is indicated for the primary immunization of individuals at risk of exposure to *Bacillus anthracis* spores in industries involved with livestock and in other circumstances where exposure might occur. Historically, the vaccine components have not been fully characterized. We have now undertaken characterization work to support on-going process validation studies. The vaccine consists of aluminum hydroxide (Alhydrogel)-adsorbed antigens obtained from culture filtrates of the non-encapsulated, toxigenic strain V770-NP-1R of *B. anthracis*. In the absence of an effective desorption procedure for the vaccine, aliquots of the fermentation filtrate prior to adsorption to Alhydrogel were subjected to Bradford protein, SDS-PAGE, Western, and ELISA analyses. Thus, a typical fermentation filtrate was determined to contain 10 μg Bradford protein per mL, > 40 % Protective Antigen (PA, based on SDS-PAGE and Western), 2-4 μg PA/ml (based on Elisa), and no detectable Edema Factor (based on Western). With regards to the Lethal Factor (LF), some LF was detected based on Western and the amount determined to be in the range of 10-30 ng LF per mL of filtrate (based on ELISA), about 1/100 of the amount of PA. To address the level of possible biological

activity of LF in the vaccine, the mouse macrophage cytotoxicity assay was performed on 11 vaccine lots. The results indicate that small molecular weight additives such as phemerol and formaldehyde have minor toxic effects on macrophage cells. However, when these were removed by dialysis, there was no toxic effect on the cells, indicating that the minor amount of LF present in the vaccine is inactive. Further characterization studies on the filtrate as well as on the final formulated vaccine are on-going.

Board 42A. In Vitro Selection and Characterization of High-Level Fluoroquinolone Resistance in *Bacillus anthracis*

L. PRICE, A. J. VOGLER, S. JAMES, P. KEIM

Northern Arizona University, Flagstaff, AZ

Two preferred therapeutics for human anthrax infections are penicillin G and ciprofloxacin. In order to characterize fluoroquinolone resistance in *B. anthracis*, we used an in vitro step-wise selection procedure to develop high-level ciprofloxacin (CIP) resistant mutants. In a series of three selection steps we were able to isolate *B. anthracis* mutants with MICs as high as 64 mg CIP/ml (>103 increase over wild-type MIC). We found that first-level mutants, selected on 0.25 mg CIP/ml, developed 1 of 4 missense point mutations in the Quinolone Resistance Determining Region (QRDR) of the gyrA gene. The most common of these mutants (Ser 85 à Leu) was used in a second selection on 1.5 mg CIP/ml. Second-level mutants developed 1 of 3 missense mutations in the QRDR of parC. The most common second-step mutant (Ser 81 à Phe) was used in a third and final selection on 24 mg CIP/ml. While some third-step mutants had an additional mutation in the gyrA gene, the majority had no alterations in any of the topoisomerase genes (gyrA, gyrB, parC and parE). Experimentally, we found no evidence for multi-drug efflux in these third-step resistant mutants. We measured mutation rates using a Luria-Delbruck design and found comparable rates to those reported in other species. These data support the "recent emergence" and/or "long dormancy" theories for the lack of genetic diversity among *B. anthracis* strains. These results also argue for the judicious use of clinically important antibiotics because of the potential risk of developing resistant pathogens.

Board 43A. Immunisation with Microencapsulated PA

L. W. J. BAILLIE[1], H. FLICK-SMITH[1], J. E. EYLES[2], J. MILLER[1], H. O. ALPAR[3], **E. D. WILLIAMSON**[1]

[1]CBD Porton Down, Salisbury, UNITED KINGDOM; [2]Dept. Pharmaceutical Sciences, Aston University, Birmingham, UNITED KINGDOM; [3]Dept. Pharamaceutical Sciences, Aston University, Birmingham, UNITED KINGDOM

Protective antgen (PA) is the key component in existing vaccines to protect against *Bacillus anthracis*, the causative organism of anthrax. There are two major goals in anthrax vaccine R&D. The first is to exploit efficiently the protective action of PA and the second is to enhance the protection achievable by mucosal immunisation against pulmonary anthrax. We have observed that nasal immunisation is particularly effective at inducing mucosal immunity in the lung. Nasal immunisation with PA can be facilitated by the encapsulation of the protein in polymeric microspheres which not only protects the protein from degradation but which also allows sustained release of the protein in the vaccinee, thus reducing the need for frequent boosting. By rendering the protein a particulate antigen, it maximises its immunogenicity. In the current study, we have investigated the optimum presentation of rPA in a microencapsulated formulation, in a two-dose immunisation regimen in the mouse model. Data will be presented which indicates that microencapsulated PA dosed on two occasions either nasally or intra-muscularly is fully protective against 10e3 median lethal doses of STI injected spore challenge in the A/J mouse. Equivalent protective efficacy can be achieved by priming mice i.m. with microencapsulated PA followed by nasal boosting with the same preparation.

Genomics and Gene Regulation
Poster Session IV – P4

Tuesday, 5:30 p.m.–6:30 p.m. | McDowell Hall

Board 19A. Siderophore Biosynthesis in *Bacillus anthracis*

S. R. CENDROWSKI, P. C. HANNA

University of Michigan, Ann Arbor, MI

Bacillus anthracis is the infectious agent of anthrax, an acute bacterial disease of the skin, respiratory and intestinal tracts. *B. anthracis* may utilize a siderophore to scavenge iron after germination in the human host. The presence of a colored complex, putatively an iron chelate, has been observed in the supernatants of *B. anthracis* cultures grown under conditions that favor production of known anthrax virulence factors. The importance of siderophores in anthrax infection may be at the initial stages during replication in the macrophage and/or in later stages during growth in the host blood. The *Bacillus* subtilis operon, dhbACEBF, encodes five biosynthetic enzymes involved in production of 2,3-dihydroxybenzoate that chelates iron by a catechol moiety. Genetic sequence data available from the *B. anthracis* genome project suggests that a dhb operon homolog exists in anthrax. The *B. anthracis* dhbCEBF homologs have been cloned. These open reading frames appear to be arranged in an operon in *B. anthracis* as well. Experiments are proceeding to detect a *B. anthracis* dhbA homolog and a dhb promoter region. A knockout construct of the dhbB homolog has been prepared and this assemblage has been mobilized into *B. anthracis* for preparation of a mutant. This dhbB mutant strain will then be utilized in experiments to examine the effects of siderophore production on survival and virulence in vitro and in vivo.

Board 22B. Development and Analysis of a *B. anthracis* Plasmid Gene Expression Assay.

J. PANNUCCI, E. WILLIAMS, R. CARY, P. PARDINGTON, R. OKINAKA, C. R. KUSKE

Los Alamos National Lab, Los Alamos, NM

Little is known about the function of the *B. anthracis* virulence plasmid, pX01, beyond the anthrax toxin genes and their known regulators. Database searches (Okinaka et al. 1999) reveal approximately 25% of the predicted pX01 ORFs have putative functions assigned, and it is uncertain whether the majority of predicted ORFs are functioning genes. Through a series of experiments, protocols were developed and modified

for isolation of RNA and fluorescence-based microarray hybridization of *B. anthracis* cDNA probes against the entire set of predicted pX01 ORFs. RNA was isolated from cells grown in various culture conditions and the results of the microarray experiments were analyzed. Our objective was to confirm expression of the predicted ORFs where possible, and determine the factors driving gene expression. Preliminary results indicated expression of pX01 ORFs other than the known virulence genes; some in a culture condition dependent manner. Comparison of expression data with pX01 DNA sequence conservation among Bacilli may suggest genes uniquely associated with the disease, anthrax.

Board 24B. Whole-genome Sequencing *Bacillus thuringiensis* Strain 97-27, a Close Relative of *Bacillus anthracis*

R. T. OKINAKA, J. LONGMIRE, R. SVENSSON, N. DOGGETT, D. BRUCE, M. MUNDT, P. J. JACKSON

Los Alamos National Laboratory, Los Alamos, NM

We have initiated the whole-genome sequencing of putative *Bacillus thuringiensis* (BT), strain 97-27. This particular strain was isolated from the wound of a French soldier serving in the war in Bosnia [New Scientist, 29 May 1999]. Other strains of BT are commonly used as a natural pesticide and classification of this strain as a pathogen has raised questions about the use of this organism as a pesticide in the US and Europe. Recent data in our laboratory indicates that strain 97-27 is more closely related to *B. anthracis* than to all of the known pesticide strains of BT. The sequencing strategy to assemble the strain 97-27 genome is based on the whole-genome, shotgun approach that has been successfully applied to the sequencing and assembly of many microbial genomes [Fleischman et al. (1995) Science, 269:496]. The project consists of two phases: a shotgun phase involving the sequencing of small insert clones to obtain 6X coverage of the 5 mbp genome and a finishing phase consisting of assembly, sequence and physical gap closure and annotation. In this report we present our initial bulk sequencing data and initial direct sequence comparisons between BT strain 97-27 and the genome of its close relative, *Bacillus anthracis* (T. Read, TIGR).

Board 26B. Cereolysin Operon of *Bacillus anthracis*

K. V. KIRILL[1], A. P. POMERANTSEV[2], S. H. LEPPLA[1]

[1]National Institute of Dental Research, Bethesda, MD; [2]State Research Center for Applied Microbiology, Obolensk, RUSSIAN FEDERATION

Although closely related on the genomic level to the highly hemolytic *Bacillus cereus*, *Bacillus anthracis* is non-hemolytic. Hemolysis by *B. cereus* results from secretion of cereolysin, which consists of phospholipase C (Plc) and sphingomyelinase(Sph) proteins encoded by the CerA and CerB genes, respectively, which are organized in an operon under the control of a PlcR-responsive promoter (Agaisse et al., Mol. Microbiol. 32:1043-1053, 1999). The *B. anthracis* genome contains a highly similar *cerAB* operon, but this appears to be transcriptionally silent, possibly because the PlcR gene of *B. anthracis* is truncated (Agaisse, ibid).

In this work we tested the enzymatic activities of the CerA and CerB proteins from *B. anthracis* and measured the binding of the PlcR protein to the *cerAB* promoter. For this purpose, the *cerA, cerB,* and *plcR* genes from *B. anthracis* and *B. cereus* were cloned and the corresponding proteins were expressed and purified as his-tagged derivatives in an *E. coli* T7 expression system. The enzymatic and hemolytic activities of CerA and CerB were estimated. Surprisingly, we found that CerB isolated from either bacterium produced hemolysis of sheep blood in the absence of CerA. CerA, hemolytically inactive alone, augmented CerB hemolytic activity. Further analysis showed that the CerB enzyme from both bacteria possesses both Sph and Plc activities, whereas CerA has only Plc activity. To determine whether the inactivity of *cerAB* in *B. anthracis* is related to the small sequence difference of the promoter from that in *B. cereus* cerAB, we performed gel-shift assays. The *B. cereus* and *B. anthracis* promoter sequences were probed with the *B. cereus* PlcR protein. The single G to T nucleotide exchange in the putative PlcR binding site did not lead to significant differences in PlcR binding. We are now testing whether the PlcR defect in *B. anthracis* can be complemented by the *B. cereus* PlcR. Thus these data demonstrate that CerA and CerB proteins of *B. anthracis* are functionally active and similar to their *B. cereus* homologues. Further work to explain the differences in hemolytic activity between these closely related bacteria will focus on gene regulation and the role of PlcR.

Molecular Interactions of Anthrax Proteins
Poster Session IV – P5

Tuesday, 5:30 p.m.–6:30 p.m. | McDowell Hall

Board 28B. Role of Protective Antigen Oligomerization in Anthrax Toxin Action

J. MOGRIDGE, M. MOUREZ, B. LACY, R. COLLIER

Harvard Medical School, Boston, MA

The assembly of anthrax toxin begins with protective antigen (PA) binding a cellular receptor and being cleaved into two fragments by a cellular protease. PA63, the fragment that remains associated with the cell, oligomerizes into heptamers and binds the enzymatic moieties, edema factor (EF) and lethal factor (LF). Oligomerization of PA63 triggers endocytosis, and the low pH environment of the endosome initiates the insertion of PA63 into the membrane and the translocation of EF and LF. We have studied the role of oligomerization of PA63 in the intoxication process by constructing two classes of mutants that do not oligomerize. Each class has one wild-type and one mutated oligomerization surface; the classes differ by which of their surfaces is competent for oligomerization and which is defective. Dimeric PA63 can be formed by mixing the two classes of mutants on cells, because their complementary wild-type oligomerization surfaces interact and their mutant surfaces prevent further oligomerization. We have found that oligomerization-defective mutants by themselves do not associate stably with the PA-binding domain of lethal factor (LFn). Dimeric PA63 does bind LFn, but can not mediate its translocation. Previous studies have shown that oligomerization is required to trigger endocytosis of PA, and we are currently assessing whether dimerization is sufficient for this to occur. In sum, oligomerization of PA63 is required for at least three steps of anthrax toxin action: binding of the enzymatic moieties, endocytosis, and translocation.

Board 30B. Development and Application of a Chromatographic Method for the Analyses of Recombinant Protective Antigen, Recombinant Lethal Factor and Lethal Factor Fusion Proteins.

B. XU, A. M. LOWE, G. YAN, D. T. BEATTIE

AVANT Immunotherapeutics, Needham, MA

Bacillus anthracis produces two toxins, edema toxin and lethal toxin. Lethal toxin is composed of two proteins, protective antigen (PA, 83 kDa) and lethal factor (LF, 90 kDa), whereas edema toxin is composed of PA and edema factor (EF, 89 kDa.) In the process of intoxication, PA binds to an as-yet undefined receptor on the cell surface and facilitates translocation of EF or LF into the cytosol. In addition, PA can translocate non-toxic derivatives of LF which contain heterologous sequences (LFn fusion proteins), thereby creating a generalized mechanism for delivery of peptide epitopes into the cytoplasm. There have been very few reports of chromatographic methods designed to analyze these proteins, and such methods would be required for the development of subunit vaccines for anthrax as well as therapeutics based on PA-mediated epitope delivery. Here we describe the optimization of a reversed-phase high performance liquid chromatography (RP-HPLC) method which uses a PerSeptive Poros R1/M column and a 2-propanol/water gradient. It is used for the determination of the concentration of rPA, LF or LFn fusion proteins in culture supernatant and in-process samples from subsequent purification steps. The method also allows determination of protein purity of these samples. Moreover, the nature of the interaction of these proteins with the column matrix give clues to their strongly hydrophobic character.

Board 32B. Specific Targeting of the Anthrax Protective Antigen to the GM-CSF Receptor

M. OSORIO, S. H. LIU, S. H. LEPPLA

NIDCR/NIH, Bethesda, MD

Protective antigen (PA) is the receptor-binding component of anthrax toxin. PA binds to an unknown receptor on the surface of cells. Upon binding to the receptor, PA is cleaved by a furin-like protease and subsequently oligomerizes to form a heptameric complex. The heptamer is the structure that transfers the catalytic subunits lethal factor (LF) and edema factor (EF) to the cytosol of the target cell. The three-dimensional structure of PA has been solved and it consists of four folding domains. Previous studies have localized the receptor-binding properties of PA to domain 4. We thus generated two fusion proteins of PA with the granulocyte-monocyte colony stimulating factor (GM-CSF) ligand. One of these consists of full length PA fused to the GM-CSF ligand and the other of PA, lacking domain 4 (truncated at aa 610), fused to the GM-CSF ligand. These fusion proteins expressed in *E. coli* as inclusion bodies were solubilized with 8 M urea and refolded in the presence of oxidized glutathione to promote the formation of disulfide bridges present in the GM-CSF ligand. We performed cytotoxicity assays on RAW264.7 cells using the fusion proteins. The full length PA fused to the GM-CSF ligand exhibited toxicity with similar kinetics to unmodified PA. However, addition of an uncleavable PA as a competitive inhibitor significantly reduced the toxicity of this fusion protein, indicating that toxicity was mainly due to the fusion protein binding to the natural receptor of PA. We are determining the extent of the contribution GM-CSF receptor binding to toxicity exhibited by the fusion protein. The fusion protein lacking domain 4 of PA was not toxic to the cells even at concentrations as high as 3000 ng/ml. We have shown that this fusion protein is able to efficiently bind to cells displaying a high density of GM-CSF receptors on the cell surface. However, the fusion protein is not cleaved by a cell surface-associated furin-like protease. Work is underway to determine if binding is exclusively through the GM-CSF receptor and to determine the reason for the inability of this fusion protein to be cleaved on the cell surface. The ability to target PA to specific receptors will provide a powerful tool for the delivery of toxins such as LF or LF fusion toxins to specific cell types.

Board 35A. Neutralizing Monoclonal Antibodies Directed against PA

F. BROSSIER, A. LANDIER, O. JEANNEQUIN, M. MOCK

Institut Pasteur, Paris cedex 15, FRANCE

Two monoclonal antibodies, 7.5 and 48.3, directed against PA and neutralizing the anthrax toxin activities were produced. The neutralizing activity of 48.3 is 10 times higher than that of 7.5. PA was cleaved by chymotrypsin and by trypsin to generate specific polypeptides. Using these fragments and mutant proteins deleted in the carboxyterminal domain of PA, we have shown that 7.5 interacts with an epitope localized in domain 4. The antibody 48.3 recognized an epitope localized in the region between positions 313 and 608 which includes part of domain 2 and entire domain 3. We determined that the antibody 7.5 prevents the binding of PA to its cell receptor. The antibody 48.3 does not recognized PA63 but prevents the cleavage of PA at the RKKR site, thus abolishing the interaction with LF. This suggest that 48.3 binds to a conformational epitope present in PA but absent in PA63.

We tested the capacity of the monoclonal antibodies to neutralize the activities of the toxins produced by the Sterne strain *in vivo*. We showed that the two antibodies prevented edema formation and death of animals after a lethal challenge with spores of the Sterne strain.

This study contributes to the functional analysis of PA and suggests that monoclonal antibodies 7.5 and 48.3 could be used in therapeutics, in combination with antibiotics, to prevent both toxemia and septicemia.

Board 37A. Random Mutagenesis to Study Lethal Factor (LF):Mitogen Activated Protein Kinase Kinase 1 (MEK1) Interaction

A. P. CHOPRA, N. S. DUESBERY

Van Andel Research Institute, Grand Rapids

Bacillus anthracis, the etiological agent of anthrax, produces an exotoxin consisting of protective antigen, lethal factor (LF) and edema factor (EF). EF is a Ca^{2+}/calmodulin-dependent adenylate cyclase which causes a dramatic increase in cellular levels of cAMP, whereas LF is Zn^{2+}-dependent protease that has been shown to cleave members of the mitogen activated protein kinase kinase (MEK) family. To identify elements of LF involved in MEK interaction, we randomly mutagenized select regions of the LF gene by PCR amplification in the presence of the nucleoside analogue 6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-C][1,2]oxazin-7-one(dPTP) and/or 8-oxo-2'deoxyguanosine (8-oxo-dGTP). The complete LF gene was regenerated in either two or three fragment overlapping PCR. Reaction mixtures containing dPTP and/or 8-oxo-dGTP generated an extensive array of transition and transversion mutations (A→G, T→C, A→C, A→T, G→C, G→T) and codon

change in the absence of insertion and deletion. The extent of mutation depended on the concentration of base analogue used. The LF gene was cloned in the pSJ115 vector and transformed into non-sporulating strain of *B. anthracis* BH445. Preliminary analyses with unpurified supernatants indicates a measurable change in LF toxicity. The purified proteins will be assessed for their ability to bind and cleave MEK *in vitro*. The effect of these and other previously characterized mutations on LF-MEK interaction will also be discussed.

Immunoprophylaxis and Treatment

Poster Session IV – P6

Tuesday, 5:30 p.m.–6:30 p.m. | McDowell Hall

Board 3A. A High-throughput Elution ELISA for the Measurement of High Avidity Antibodies against Protective Antigen Produced by *Bacillus anthracis*

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[1]CAMR, Salisbury, UNITED KINGDOM; [2]Battelle Memorial Institute, Columbus

Protective antigen (PA) is one of the principal toxin components of *Bacillus anthracis* and is generally considered to be the dominant protective antigen present in the current manufactured anthrax vaccine preparations. Protective immunity can be conferred experimentally by passive transfer of polyclonal antibodies to PA. However, no specific correlates for immunity against *Bacillus anthracis* have been identified. The presence of high-avidity antibodies, induced on avidity maturation, has been previously demonstrated to correlate with protection against conjugate vaccines, for example against Haemophilus influenzae type B. Here we describe a thiocyanate elution ELISA for the measurement of high avidity antibodies against PA induced by vaccination, and its application for the assessment of serum samples using a robotic ELISA platform.

Board 5A. Interlaboratory Comparison of Methodologies to Enumerate Viable *Bacillus anthracis* Spores

A. J. PHIPPS[1], B. E. IVINS[2], P. F. FELLOWS[2], R. E. BARNEWALL[1], C. BOTEZAN[3], S. RASTOGI[3], R. MYERS[3]

[1]Battelle, Columbus, OH; [2]USAMRIID, Fort Detrick, MD; [3]BioPort Corporation, Lansing, MI

The licensed potency test for the Anthrax Vaccine Adsorbed (AVA) requires challenge of vaccinated guinea pigs with a specified number of viable *Bacillus anthracis* spores. Historically, both the spread and pour plate techniques have been used to enumerate viable *B. anthracis* spores. Both techniques are based on the premise that each viable spore, upon germination, will form a single bacterial colony that is visible to the naked eye. However, the accuracy of the pour plate technique may be more sensitive to variations in media sources and preparation techniques as compared to the spread plate technique. The study was designed to evaluate the effect of incubation temperature, media sources, media preparation, and sample handling upon the spread and pour plate techniques performed by three laboratories. Samples of a working *B. anthracis* spore suspension (WSS-1) prepared at BioPort Corporation were sent to the United States Army Research Institute of Infectious Diseases (USAMRIID) and to Battelle Memorial Institute. USAMRIID and BioPort Corp. used both the spread and pour plate techniques to enumerate the number of viable spores in the WSS. Additionally, Battelle used the spread plate technique to enumerate the number of viable spores in the WSS. The effect of incubation temperature, media source, media preparation, and sample handling upon the accuracy of the spread and pour plate techniques was compared between laboratories. Additionally, spread plate results obtained by all three laboratories were compared. In conclusion, the pour plate technique appears to be less rugged and robust than the spread plate technique. Furthermore, the intermediate precision of the spread plate technique was determined between laboratories.

Board 6A. Efficacy of a Recombinant PA Produced in *Bacillus brevis* against *Bacillus anthracis* Spore Challenge in Guinea Pigs

H. B. OH, Y. M. PARK, J. S. HAN, W. K. SEONG

Laboratory of Bacterial Toxins, Department of Microbiology, National Institute of Health, Eunpyung-gu, Seoul, 122-701, REPUBLIC OF KOREA

To develop a mass production system for the protective antigen (PA) of *B. anthracis*, a recombinant secreting PA was constructed using *B. brevis*-pNU212 system. The recombinant secreted about 300 μg of recombinant PA (rPA) per ml of 5PY-Em medium after 4 days incubation at 30°C. The rPA was purified by FPLC. Purified rPA had the same N-terminal amino acid sequences as those of PA from *B. anthracis* ATCC 14185. The biological activity of recombinant PA was confirmed by J774A.1 cell lysis assay. The protective efficacy of immunization against anthrax with rPA combined with adjuvant including alum gel, ribi-trimix was tested in female guinea pigs. In guinea pigs, adjuvants enhanced antibody titers to PA as well as survival after an intramuscular challenge of virulent *B. anthracis* ATCC 14578 and K-Yubudol spores. Immunization with rPA without adjuvant showed 1:8,148 antibody titer to PA. And immunization with rPA in combination with alum gel and ribi-trimix revealed 1:42,271, 1:113,609 antibody titer, respectively. In neutralization assays, sera from guinea pigs immunized with rPA without adjuvant showed 1:352 antibody titer. Using alum gel and ribi-trimix as adjuvant, neutralizing antibody titer were 1:514 and 1:6,400, respectively. Guinea pigs immunized without adjuvant had an incomplete protection against two virulent spores. In the case of alum gel and ribi-trimix, guinea pigs immunized with rPA showed 100% protection from intramuscular challenge with $50LD_{50}$ spores of *B. anthracis* ATCC 14578 and K-Yubudol.

Board 8A. Multi-color Flow Cytometric Analysis for Simultaneous Mapping of the Antibody Response to Multiple Epitopes of the Protective Antigen of *Bacillus anthracis*

D. S. REED, S. LITTLE, J. SMOLL

USAMRIID, Frederick, MD

A flow cytometry-based microsphere assay has been developed to detect and measure neutralizing antibodies in sera to protective antigen (PA) of *B. anthracis*. Monoclonal antibodies 14B7 and 2D3, which neutralize PA in vitro, were conjugated with fluorescein isothiocyanate (FITC). Both of

these FITC-conjugated mAbs were capable of recognizing PA covalently bound to microspheres. Unlabeled 14B7 inhibited with the binding of FITC-14B7 but not FITC-2D3. The reverse was true for unlabeled 2D3. The competition between the unlabeled and labeled mAb could be titrated by incubation with increasing dilutions of the unlabeled mAb. Sera from AVA-vaccinated animals contained antibodies that competed with the FITC-14B7 and FITC-2D3. Using a standard curve developed with the unlabeled mAbs it was possible to measure the amount of antibodies in the sera of AVA-vaccinated animals that competed with FITC-14B7 and FITC-2D3. By comparison with the total amount of IgG against PA in the rabbit sera the relative contribution of antibodies competing with 14B7 and 2D3 could be determined. Antibody against the epitope recognized by 14B7 appears to be dominant, comprising 26% of the antibody response in rabbits that received the full human dose of AVA. Only 9% of the antibody in those same animals were directed against 2D3. Preliminary analysis of the data suggests that the ratio of antibodies competing with 14B7 and 2D3 in relation to the other anti-PA antibodies present changes with the dose of AVA given to the rabbits. In rabbits given only 1:16 of the human dose, the ratio of antibody competing with 14B7 declined from 26% to 21%. The data obtained thus far indicate that this new microsphere assay will allow for a better determination of a functional antibody response that may predict vaccine efficacy in humans against exposure to aerosolized *B. anthracis*.

Board 10A. An Immunogenicity and Reactogenicity Evaluation of a Recombinant *Bacillus anthracis* Protective Antigen Vaccine

L. J. THOMAS, M. D. PICARD, A. M. LOWE, B. XU, L. M. GARRETT, L. A. MOORE, D. T. BEATTIE, A. SCORPIO

AVANT Immunotherapeutics, Needham, MA

A study was conducted with a recombinant *Bacillus anthracis* protective antigen (PA) vaccine, lacking an adjuvant, to determine its immunogenicity and reactogenicity in New Zealand White (NZW) rabbits. Recombinant PA was produced in *E. coli*, purified to > 95% homogeneity and characterized. In this study, three NZW rabbits were immunized with 0.05 mg recombinant PA in saline, without adjuvant, on days 1, 15 and 29, intramuscularly in the thigh, alternating sides for each successive dose. Reactogenicity was determined by observing the injection site for seven days following each of the injections, grading the observations on a scale of 0 to 4, from "none" to "severe". Blood samples were taken on days 1 (preimmunization), 14, 28, 42, 70 and 90, and processed as serum. All injection site observations were determined to be "none" (grade 0, normal color and no swelling), except for one observation of "minimal" (grade 1, light pink indistinct erythema, slight swelling with an indistinct border) in one rabbit on one day immediately following an injection. Immunogenicity was determined by measuring antibodies specific for PA by ELISA. On day 28, following two immunizations, the three rabbits developed an average anti-PA titer exceeding 1/250,000. By day 42, following all three immunizations, the antibody titer of the rabbits was maintained in a range of 1/128,000 to 1/500,000. In conclusion, this study demonstrated that the administration of three doses of this recombinant *Bacillus anthracis* protective antigen (PA) vaccine, without adjuvant, produced specific, high titered anti-PA responses, with virtually no observed reactogenicity.

Board 12A. Intracellular Detection of PA-specific IL4 following Immunisation with a Novel and Current Anthrax Vaccines in Rhesus Macaques.

C. A. ROWLAND, E. D. WILLIAMSON, S. M. JONES

DERA, CBD Porton Down, Salisbury, UNITED KINGDOM

Anthrax is a fatal disease caused by the gram-positive bacteria, *Bacillus anthracis* (1). Protective anthrax vaccines currently licensed in the UK and US may cause side effects in some individuals, are expensive to produce and require several doses to ensure complete protection (2). Therefore, a second generation vaccine is under development containing a recombinant form of the protective antigen (PA) from *Bacillus anthracis*, the principal protective antigen in immunity to anthrax (3). Antigen-specific lymphocyte proliferation assays in mice and rhesus macaques (*macaca mulatta*) show that production of anti-rPA antibodies is T-cell dependent (4). However, the T cell response to PA has currently not been fully characterised. CD4+ T helper (Th) cells or CD8+ cytotoxic T (Tc) cells produce distinctive cytokine profiles that enable them to function appropriately in response to antigen. These subsets maybe divided into Th1 or Tc1 and Th2 or Tc2 subsets (5). The cytokines IFN-γ and IL4 are expressed by different T cell subsets (T1 and T2 respectively) and are generally considered to be mutually exclusive (5). Their production and subsequent detection, therefore, can be used to determine the type of immune response elicited in response to antigen. We obtained peripheral blood samples from Rhesus macaques immunised during preclinical trials with several concentrations of rPA adsorbed to alhydrogel. UK and US licensed vaccines were also administered to allow deduction of comparative efficacy with the new vaccine. Using flow cytometry, we aimed to study immune responses to the vaccines in immunised animals by determining T cell activation marker expression and intracellular IL4 and IFN-γ production following *in vitro* stimulation with rPA. Specific intracellular IL4 production was detected in CD4+ T cells following rPA *in vitro* stimulation of blood from Rhesus macaques immunised with the current US, UK and a novel vaccine (25μg/ml rPA). This suggests that Th2-biased immunity is elicited in immunised animals in response to *in vitro* rPA stimulation. IL4 was not detected in CD8+ T cells indicating that cytotoxic T cells were not involved in cell-mediated immunity to the vaccines. CD69 expression on CD8- cells and IL4 production in CD4+ T cells appeared to be correlated suggesting that CD69 maybe a good marker of activation/proliferation for rPA. 1. Turnbull PC (1998) Anthrax Zoonoses Textbook Oxford University Press (Chap 1) p3-5 2. Fritz DL, Jaax NK, Lawrence WB (1995) Lab Invest 73 691-702 3. Dykhuizen M et al (2000) Cytometry 40 p69-75 4. McBride BW et al (1998) Vaccine 16 p 810-817 5. Mosmann TR & Sad S (1996) Immunology today 17(3) 138-146

Board 14A. In vitro Assays for Acellular Anthrax Vaccine Analyses

B. HALLIS, C. QUINN, N. SILMAN, A. ROBERTS, S. HISCOTT, G. LLOYD, M. HUDSON

CAMR, Salisbury, UNITED KINGDOM

Characterisation and evaluation of the current UK licensed human anthrax vaccine require a variety of in vivo tests to determine protective efficacy of the vaccine. A programme of alternative assessment strategies, based on novel in vitro technologies, has been developed. These technologies may

technologies, has been developed. These technologies may facilitate Quality Control for the continued production of consistent and effective vaccine. The major vaccine constituents are considered to include the individual toxin components: protective antigen (PA), lethal factor (LF) and oedema factor (EF) and their binary toxins: lethal toxin (LT) and oedema toxin (ET) precipitated onto aluminium salts. Each or all such components may contribute individually or collectively to the potency, toxicity or reactogenicity of the vaccine. This presentation describes the development and application of sensitive and quantitative functional assays that can discriminate between the binary anthrax toxins (LT and ET) and their individual protein components (LF, EF and PA). These in vitro assay systems are based on a combination of cell receptor binding and biological activity. Rapid, sensitive and specific immunoassays for the detection of PA, LF, EF and the two major S-layer proteins (Sap and EA-1) are also described.

Board 16A. Evaluation of the Therapore(TM) Antigen Delivery System as an Anthrax Vaccine and Adjuvant for Heterologous Antigens

A. SCORPIO, K. P. KILLEEN, M. D. PICARD, D. T. BEATTIE, A. LOWE, B. XU, L. GARRETT, L. MOORE, L. J. THOMAS

Avant Immunotherapeutics, Needham, MA

TheraporeTM is a protein-based antigen transport system, consisting of two components: *Bacillus anthracis* protective antigen (PA) and the N-terminal 254 amino acids of *B. anthracis* lethal factor (LFn). PA serves as a docking protein for LFn, facilitating the endosomal entry of both components into cells, where processing occurs for antigen presentation. T cell responses can be elicited in mice against epitopes or large antigens by fusing them to LFn and administering the fusion protein product along with PA. In the current study we evaluated the potential of the TheraporeTM antigen transport system to serve as an anthrax vaccine as well as an adjuvant for heterologous antigens fused to LFn. Rabbits were immunized with PA, LFn, or both PA and LFn concurrently, given a homologous boost at 2 and 4 weeks, and bled at 0, 4 and 6 weeks. Serum was assayed for total IgG by ELISA. Antibody titers against PA were as high as 1/500,000 in rabbits immunized with either PA alone or PA plus LFn. Titers against LFn were five fold higher in rabbits immunized with PA plus LFn compared with LFn alone (1/125,000 versus 1/25,000). The potential of using the TheraporeTM system to enhance the immune response against heterologous antigens was examined by fusing the outer surface protein A (OspA) of Borrelia burgdorferi to LFn, creating LFn-OspA. BALB/c mice were immunized with LFn-OspA or LFn-OspA plus PA, given a 2 week homologous boost, and bled at 0,4, and 6 weeks. Titers against OspA in mice administered LFn-OspA plus PA were about 5 fold higher than titers in mice administered LFn-OspA alone (1/15,000 versus 1/3,000). The study demonstrates the utility of the TheraporeTM system as a potential anthrax vaccine as well as an adjuvant for heterologous antigens.

Board 39B. Improvements in the Development of Live Salmonella Vectors for the Delivery of Vaccine Candidates against *B. anthracis*

W. BEYER[1], K. E. GRIFFIN[2], H. S. GARMORY[2], R. W. TITBALL[2]

[1]University of Hohenheim, Institut for Environmental and Animal Hygiene, Stuttgart, Garbenstraße 30, 70599, GERMANY; [2]Defence Science and Technology Laboratory, CBD, Porton Down, Salisbury, Wilts., SP4 0JQ, UNITED KINGDOM

Live Salmonella carrying the Protective Antigen gene in its full or proteolytically-processed form (PA83 or PA63) have been constructed. The procaryotic expression cassettes encodes the appropriate PA63 or PA83 coding sequence, which was designed and resynthesised for high level expression of the gene in *E. coli.* The encoded protein was fused to the Hly-export cassette of *E. coli* to facilitate the export of the fusion protein. This construct was introduced as a plasmid or into the chromosome of a commercially available vaccine strain of S. typhimurium (strain Zoosaloral). In parallel, a set of eukaryotic expression cassettes was constructed expressing the PA gene or an antigenic domain of PA only into various compartments of the cell. These constructs may be appropriate as DNA-vaccine candidates which could be administered as naked DNA, encapsulated DNA or with live Salmonella as a carrier. Bacteria carrying the procaryotic export cassette with PA83 or PA63 extrachromosomally were given orally or i. v. to A/J mice in a three dose vaccination schedule. The IgG response was determined and mice responding well will be challenged with *B. anthracis* STI strain. Preliminary results of those and further trials will be presented as far as available.

Board 40B. Anthrax Vaccine: Safety and Antibody Response of a Dose-Reduction, Route Comparison Study In Humans

P. R. PITTMAN, G. KIM-AHN, D. Y. PIFAT, K. COONAN, P. GIBBS, S. F. LITTLE, J. G. PACE-TEMPLETON, R. MYERS, G. W. PARKER, A. M. FRIEDLANDER

USAMRIID, Frederick, MD

Background: Anthrax Vaccine, Adsorbed (AVA), an effective countermeasure against anthrax, was administered as 6 subcutaneous (SQ) doses over 18 months. To optimize the vaccination schedule and route of administration, we performed a prospective pilot study comparing the use of fewer doses administered intramuscularly (IM) and SQ with the current schedule and route.

Methods: We enrolled 173 volunteers, randomized to 7 groups, who were given AVA once IM or SQ; 2 doses, 2 or 4 weeks apart, IM or SQ; or 6 doses at 0, 2, 4 weeks and 6, 12, and 18 months (control group, licensed schedule and route). Systemic and local reactions were evaluated and recorded. Anti-protective antigen (PA) IgG concentrations were determined by using a validated enzyme-linked immunosorbent assay (ELISA) and were correlated to a validated toxin neutralization test on a subset of samples.

Results: Systemic adverse event occurrences were independent of route or gender. Injection site erythema and induration occurred more commonly when the vaccine was administered SQ (P

The IgG anti-PA concentrations two weeks after 2 AVA doses given 4 weeks apart, IM or SQ, were comparable to those two weeks after 3 AVA doses SQ given 2 weeks apart (p = 0.615). The distribution of peak (week 6) IgG anti-PA antibody concentration did not differ among the 0-4 IM, 0-4 SQ, and the control groups (p = 0.996). Antibody response rates (>25ug/ml) for these groups were 96-100%.

Conclusions: IM administration of AVA was associated with fewer local reactions than SQ administration and reaction rates decreased with a longer dose interval between the first 2 doses. The peak anti-PA IgG antibody response of subjects given 2 doses of AVA 4 weeks apart was comparable to that seen among individuals who received 3 doses of AVA at 2-week intervals.

Board 41B. Preparation of a Freeze-dried Anthrax Vaccine Reference Standard for Use in the Guinea Pig Potency Test

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[1]National Institute for Biological Standards and Control, Potters Bar, UNITED KINGDOM; [2]Centre for Applied Microbiology and Research, Porton Down, UNITED KINGDOM

Aim: The potency of new Anthrax vaccine batches is determined in guinea pigs challenged with virulent *Bacillus anthracis*. The level of protection afforded by new vaccine batches has to be greater or equal to the level of protection offered by the standard, a previously produced Anthrax vaccine batch held at 2-8 oC. Prolonged storage (>4 yrs) of this standard is likely to cause degradative changes to vaccine antigens, which may affect potency of the standard and therefore the interpretation of test results of new batches. Ideally a reference standard should have stable characteristics to allow comparison with new vaccine batches. An Anthrax vaccine batch produced in 1997 was freeze dried to prepare a stable reference standard. Over a two year period, the potency of the freeze-dried vaccine preparation will be tested in guinea pigs alongside the current standard. This will reveal if the vaccine remains stable.

Results: The results of two studies will be presented. The first study showed that addition of cryopreservants such as trehalose, sucrose and dextran did not have a beneficial effect on the potency of freeze-dried vaccine. The freeze-dried vaccine (with or without additives) retained its potency after a two-month storage period at temperatures ranging from -20oC to +56oC. In the second study, the stability of the freeze-dried vaccine (without additives) is investigated for the duration of at least two years. So far, results indicate that the vaccine remains stable after storage at -20oC or +20oC for 9 months, whereas its potency declined significantly after storage at +56oC for 4-9 months.

Discussion: The availability of a stable reference standard should improve the quality of the anthrax vaccine potency test. In addition, the standard will allow comparison between different potency test formats and different Anthrax vaccine formulations. Our initial results indicate that production of such a standard is feasible.

Board 42B. Protection Against Lethal Anthrax Toxin Challenge by Genetic Vaccination with Plasmids that Encode *B. anthracis* Protective Antigen and/or a Mutant Form of Lethal Factor

B. PRICE[1], A. LINER[1], S. PARK[2], S. H. LEPPLA[3], D. R. GALLOWAY[2]

[1]The Ohio State University, Columbus, OH; [2]NIDCR, NIH, Bethesda, MD; [3]BDRD, Naval Medical Research Institute, Bethesda, MD

The ability of genetic vaccination to protect against a lethal challenge of anthrax toxin was evaluated. Balb/c mice were immunized via gene gun inoculation with eucaryotic expression plasmids encoding either the protective antigen (PA), a mutated form of lethal factor (LF) or a combination of the two. Plasmid pCLF4 contains the N-terminal region (aa 10-254) of *B. anthracis* lethal factor (LF) cloned into the pCI expression plasmid (Promega). Plasmid pCPA contains a biologically active portion (aa 175-764) of *B. anthracis* protective antigen (PA) cloned into the pCI vector. Plasmid preparations were coated onto 1 micron gold particles and injected via gene gun (1 ug plasmid DNA/injection) three times at two week intervals with either pCLF4, pCPA, or a 1:1 mixture of each plasmid. Sera was collected and analyzed for antibody titer as well as antibody isotype. Significantly, antibody titers from mice immunized with the combination of pCLF4 and pCPA were four to five times greater to either PA or LF than mice immunized with either gene alone as determined by ELISA. Two weeks following the final boost, all mice were challenged with a lethal dose (5 x LD50) of lethal toxin (PA plus LF) injected i.v. into the tail vein. 100% of mice immunized with pCLF4, pCPA, or a combination of each plasmid were protected. These results demonstrate that DNA-based immunization alone can provide protection against a lethal toxin challenge and that DNA immunization against the LF antigen alone provides complete protection. In collaboration with DERA at Porton Down, U.K. we have recently demonstrated that mice immunized with DNA vaccines against PA and/or PA/LF survive an infection challenge with viable *B. anthracis* spores.

Immunoprophylaxis and Treatment

Wednesday, 8:30 a.m.–11:15 a.m. | Key Auditorium

Live Recombinant Anthrax Vaccines: Influence of Capsule on Protective Efficacy

J. P. BARNARD, S. A. TOBERY, D. J. CHABOT, **A. M. FRIEDLANDER**

U.S. Army Medical Research Institute of Infectious Diseases, Frederick

Efforts to create an improved anthrax vaccine that provides a high level of protection with few doses have included combining purified anthrax protective antigen (PA) with various adjuvants, use of genetic vaccination, or constructing live, recombinant vaccines using bacterial or viral vectors, including *B. anthracis,* to express the cloned PA gene. In this study, we examined the role of the poly-D-glutamic acid capsule on the protective efficacy of a live, recombinant PA-producing *B. anthracis* vaccine. The protective efficacy of two live,

recombinant anthrax vaccines, one encapsulated and the other non-encapsulated, was assessed in Hartley guinea pigs. These live vaccine strains contained the pPA102 plasmid that expresses the anthrax PA protein to a high degree. There were no significant strain-related effects on: PA production, plasmid stability *in vitro*, or *in vivo*, or survival of the vaccine strain in the host at the site of inoculation. The encapsulated strain induced a significantly greater anti-PA antibody response than did the non-encapsulated strain. More importantly, the encapsulated strain provided significantly greater protection against a high-dose challenge with *B. anthracis* spores. While the exact mechanism of enhanced protection remains to be determined, the results suggest that including the *B. anthracis* capsule may lead to the development of a more efficacious anthrax vaccine.

Protective Antigen Domains As Novel Anthrax Vaccine Candidates

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DERA Porton Down, Salisbury, UNITED KINGDOM

Bacillus anthracis Protective Antigen (PA) is essential for host cell intoxication, as it contains the host cell receptor binding site, and is also the dominant factor in stimulating both natural and vaccine induced immunity. PA is composed of four distinct functional domains. We have cloned and expressed truncates of these domains as glutathione-s-transferase (GST) fusion proteins and assessed their immunogenicity and protective efficacy against anthrax strain STI challenge in A/J mice. Antibodies to PA are essential for protection against anthrax infection, and all the truncates stimulated anti-PA IgG titres, but there was no correlation between these and the degree of protection, suggesting that the truncates also stimulate cell mediated components of the immune response. Mice were fully protected against intraperitoneal challenge with the equivalent of 10^3 MLDs of STI spores, by immunisation with truncates containing domain 4, the host cell receptor binding region. The absence of this domain resulted in breakthrough at this challenge level, implying that domain 4 is the immunodominant sub-unit of PA. Removal of the domain 1a region from the truncates resulted in lower anti-PA IgG titres and reduced protection compared to their intact counterparts, suggesting that the structural conformation of the rPA truncates is important for epitope recognition and protection. These results further elucidate the role of PA in protection against anthrax infection by showing that protection can be attributed to individual domains of PA, and highlights the potential for use of rPA domains as vaccine candidates.

Anthrax Vaccine Development Work at CBD Porton Down

LES BAILLIE

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CBD Porton Down is part of the United Kingdom's ministry of defence and is tasked with protecting the UK's armed forces against attack with chemical and biological weapons. Anthrax is a disease caused by a bacterium, *Bacillus anthracis*. Although primarily a disease of animals it can also infect man, sometimes with fatal consequences. Recent interest in the organism has centred on its potential to be employed as a biological weapon. As a consequence a considerable amount of effort has been focused on the development of vaccines which meet the standards of the 21st century. The existing current licensed anthrax vaccines may protect against infection but suffers from problems of standardisation, are relatively expensive to produce, require repeated dosing and have been associated with transient side effects. In addition little is known about the immune response induced by the existing US/UK licensed vaccines in humans. To understand better the basis of the response we have characterized the IgG antibody (ELISA) and T cell population (FACS) responses of vaccinees to a range of *B. anthracis* derived antigens (abstract 1).

.Due to the problems associated with the existing current licensed UK vaccine, DERA Porton is developing a second generation human anthrax vaccine based on recombinant Protective antigen (rPA). A high level *E.coli* based expression system has been developed and will be described (abstract 2). Research is in progress to understand the basis of the protective immune response induced by rPA with a view to identifying a surrogate marker of protection. In support of this aim we have developed a small animal model (A/J mouse) and have undertaken research to determine which regions of the PA protein (domains) are essential for protection (abstract 3). Studies have also been performed in non-human primates (Rhesus macaques) to characterise the response to rPA (dose response) and to the current UK and US vaccines (abstract 4).

Once completed the second generation vaccine will still have to be given by injection and is likely to require at least two doses to induce protection. For this reason work is in progress to develop vaccine formulations (3rd generation) that can be administered via the oral/ intranasal route (user friendly) and can induce protection following a single dose. This presentation will describe three of the approaches we have adopted; microencapsulation (abstract 5), live vector (abstract 6) and naked DNA .

Abstracts

1. Baillie *et al.,* Characterisation of the human immune reponse to the UK anthrax vaccine.
2. Walker *et al.,* Expression of *B.anthracis* PA from *E.coli*
3. Flick-Smith et al., Protective Antigens domains as novel anthrax vaccine candidates.
4. Rowland *et al.,* Intracellular detection of PA specific IL-4 following immunization with novel and current anthrax vaccines in Rhesus macaques.
5. Flick-Smith et al. Protection studies following mucosal and parenteral delivery of microsphere associated B.anthracis Protective Antigen.
6. Beyer *et al.,* Improvements in the development of live Salmonella vectors for the delivery of vaccine candidates against *B.anthracis.*

Vaccine Research at Pasteur Institute

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Anthrax affects all mammals including man. After entry into the host, the spores of *Bacillus anthracis* germinate and yield capsulated, toxin producing bacilli. The live spore vaccine, the toxinogenic non capsulated attenuated Sterne strain, is used satisfactorily for veterinary purposes, but has side effects in some sensitive species due to residual virulence. The

development of recombinant genetics in *B. anthracis* has led to the appearance of modern alternatives. We have constructed Sterne-strain derivatives producing genetically detoxified EF and LF. They offer safer, nontoxic, live vaccine candidates. Moreover the use of such strains would also prevent environmental contamination with strains harboring wild-type pXO1. These studies have also highlighted the potential of *B. anthracis* delivering foreign antigens *in vivo*. Various strategies have been used to induce specific protective immunity: Production of antigens under the control of the *pag* promoter, anchoring at the bacterial surface by fusion to the SLH motifs of EA1, and presentation to the immune system via PA through fusion to LF254 have all proven to be efficient. Moreover, the response can be directed toward humoral or cellular immunity if the recombinant strain also produces Listeriolysin O. *B. anthracis* is therefore a potential vehicle for multivalent veterinary live vaccines.

PA is the component targeted by the protective immune response, and a cell-free-PA based vaccine is used for human vaccination. However, its efficacy is far below that of the live vaccine when tested in animal models. It therefore appears that other components and/or various immune response mechanisms are probably required for efficient protection. We have shown that the addition of formaldehyde-inactivated spores (FIS) of the Sterne strain protects 100% of mice and guinea pigs against challenge with virulent *B. anthracis* strains under conditions in which PA alone is ineffective. Infection by *B. anthracis* involves both spore germination and subsequent vegetative cell multiplication. The protection conferred by FIS may act on either of these processes. We constructed a challenge strain the virulence of which is entirely due to its multiplication properties. This strain is derived from the wild-type virulent strain and carries a nonpolar deletion into the *pagA* gene encoding PA. Immunization with FIS or with PA+FIS significantly protected against infection with this capsulated PA deficient strain. The respective contribution of the immune responses to PA and to spore antigens to protection will be discussed.

Neutralizing Anti-PA Antibody Titer as a Correlate of Protective Immunity Conferred by Anthrax Vaccine

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The need to develop more efficacious Anthrax vaccines or alternative vaccination regimes underline the importance of defining reliable markers for protective immunity. In order to design such markers we have immunized guinea pigs with a Protective Antigen (PA) based vaccine using different protocols and monitored the immune response and the corresponding extent of protection against lethal challenge of Anthrax spores. Active immunization was performed by single injection using two alternative protocols: a. vaccination with decreasing amounts of PA; b. vaccination with constant amounts of PA polypeptide, using PA that was thermally inactivated for increasing period of times. In both studies a direct correlation between survival and neutralizing antibody titer was found (R^2 = 0.92, 0.95). Most significantly, in the two protocols a similar neutralizing antibody titer range provided 50% protection. Furthermore, in a complementary study involving passive transfer of PA hyperimmune sera to naive animals, a similar correlation between neutralizing antibody titers and protection was found. In all three immunization studies neutralization titers equal or above 300 were sufficient to confer protection against a dose of 40 LD_{50} Vollum spores. Such consistency in correlation of protective immunity with anti-PA antibody titers was not observed for antibody titers determined by ELISA. Taken together, these results clearly demonstrate that neutralizing antibody to PA constitutes a major component in the protective immunity against Anthrax, and suggest that this parameter could be used as a surrogate marker for protection.

Engineering Antibody Therapeutics which Neutralize Anthrax Toxin

J. A. MAYNARD[1], D. GALLOWAY[2], S. H. LEPPLA[3], B. IVERSON[1], G. GEORGIOU[1]

[1]University of Texas at Austin, Austin, TX; [2]Ohio State University, Columbus, OH; [3]National Institutes of Health, Bethesda, MD

We have developed recombinant antibodies which bind and neutralize the protective antigen (PA) of anthrax toxin and have potential for use as both diagnostic and therapeutic reagents. Efforts have been concentrated in two areas: 1) developing a rapid technology to quantitatively identify the molecular determinants of binding, and 2) increasing antibody affinity and avidity to confer improved *in vitro* and *in vivo* neutralization capabilities.

The molecular determinants of antibody binding have been determined by mutating individual amino acid residues to alanine, using a novel strategy to permit rapid structure-function studies. Briefly, small amounts of protein are produced from PCR-generated mutants in a cell-free transcription-translationreaction, followed by surface plasmon resonance analysis to provide quantitative off-rate comparisons. These data have permitted the development of a molecular model of the toxin–antibody interaction, which suggests that the 14B7 hybridoma (Little, *et al. Infection and Immunity* 56:1807-1815) and recombinant derivatives neutralize PA by binding an epitope overlapping that recognized by the cellular receptor. Binding appears to be primarily to the small loop of domain four; in particular tyrosine 688 protrudes from the loop and is complemented by a hydrophobic hole in the antibody binding site. Efforts to confirm this model by solving the structure of the toxin-antibody crystal will be reported.

Antibody affinity has been improved by directed evolution methods, selecting for reduced off-rates. Beginning with an equilibrium dissociation constant (K_D) of 10 nM, we have improved the intrinsic affinity 30-fold, to ~300 pM. Furthermore, the influence of avidity has been explored through the generation of multivalent species. For the wild-type binding site, an increase from one to three binding sites results in a 50-fold improved K_d. Multivalency combined with evolved binding sites exhibits a further decrease in the dissociation constant. Importantly, neutralization of an anthrax toxin challenge has been shown to correlate with antibody affinity in an *in vitro* cell cultureassay, and results from *in vivo* neutralization studies will be reported. A mathematical model has been developed to quantitatively predict changes in *in vivo* neutralizing ability as a function of antibody affinity and molecular size.

Notes

Notes